LYMPHOTOXIN-ß, LYMPHOTOXIN-ß COMPLEXES, PHARMACEUTICAL PREPARATIONS AND THERAPEUTIC USES THEREOF

TECHNICAL FIELD OF THE INVENTION

5   This invention relates to lymphotoxin-ß, a lymphocyte membrane-type polypeptide. Lymphotoxin-ß, also referred to as p33, has been identified on the surface of T lymphocytes, T cell lines, B cell lines and lymphokine-activated killer cells.

10   This invention also relates to complexes formed between lymphotoxin-ß (LT-ß) and other lymphotoxin-type peptides such as lymphotoxin (which we refer to herein as "lymphotoxin-α (LT-α)" to distinguish it from LT-ß) and to complexes comprising

15 multiple subunits of LT-ß. The LT-ß polypeptide of this invention is expected to be useful in holding LT-α formed within the cell on the cell surface where either LT-ß or the LT-α/LT-ß complex may act as an inflammation regulating agent, a tumor growth

20 inhibiting agent, a T cell inhibiting agent, a T cell activating agent, an immunomodulatory agent, an autoimmune disease regulating agent or an HIV regulating agent. Furthermore, the antitumor activity of the LT-α/LT-ß complex may be delivered to tumor

25 cells by tumor infiltrating lymphocytes (TILs) transfected with the gene for LT-ß.

        The invention described herein was made in part during the course of work under Grant

No. CA 35638-07-10 from the National Institutes of
Health. The U.S. Government has certain rights in this
invention.

5   The present application is a continuation-
in-part of co-pending applications PCT/US91/04588,
filed June 27, 1991 and U.S. Patent Appln. Ser.
No. 07/544,862, filed June 27, 1990.

BACKGROUND OF THE INVENTION

       The initiation of the immune response
10 involves a complex array of intercellular signals.
These signals typically involve soluble cytokines
coupled with a number of cell-cell contact dependent
signals. The contact dependent events, most notably
activation of the T-cell receptor, lend specificity to
15 the response whereas the soluble mediators are
generally responsible for maintenance of cell
differentiation and proliferation. Tumor Necrosis
Factor (TNF) and LT-α are two polypeptides generally
recognized for involvement with the initiation of the
20 immune response.

       TNF and LT-α are soluble proteins noted
originally for their ability to inhibit the growth of
tumors. [L. Old, "Tumor Necrosis Factor," Science,
230, 630 (1985)]. Further research demonstrated that
25 both proteins exhibit a wide range of activities. TNF
is synthesized in response to various inflammatory
insults by a variety of cell types including both
hematopoietic and nonhematopoietic cells, while LT-α,
in contrast, is made specifically by lymphocytes. The
30 two known TNF receptors do not appear to discriminate
between LT-α and TNF. [T. Schall et al., "Molecular
Cloning and Expression of a Receptor for Human Tumor
Necrosis Factor," Cell, 61, 361-370 (1990); C. Smith
et al., "A Receptor for Tumor Necrosis Factor Defines

an Unusual Family of Cellular and Viral Proteins,"
Science 248, 1019 (1990)]. In general, LT-α and TNF
display similar spectra of activities in in vitro
systems, although LT-α is often less potent.

5 [J. Browning et al., Studies Of The Differing Affects
Of Tumor Necrosis Factor And Lymphotoxin On The Growth
Of Several Human Tumor Lines," J. Immunol., 143, 1859
(1989)].

TNF appears to play a major role in specific
10 aspects of metabolic control, in the response to
endotoxin shock, and in the regulation of hematopoietic
cell development. [B. Beutler et al., "The History,
Properties, and Biological Effects of Cachectin,"
Biochemistry, 27, (1988); M. Akashi et al.,
15 "Lymphotoxin: Stimulation And Regulation of Colony
Stimulating Factors in Fibroblasts," Blood, 74, 2383
(1989); G. Roodman et al., "Tumor Necrosis Factor-alpha
and Hematopoietic Progenitors: Effects Of Tumor
Necrosis Factor On The Growth Of Erythroid Progenitors
20 CFU-E And BFU-E And The Hematopoietic Cell Lines k562,
HL60, And HEL Cells," Exp. Hematol., 15, 928 (1987)].

Along with IL-1 and IL-6, TNF is also a major
mediator of the inflammatory response. [D. Cavender
et al., "Endothelial Cell Activation Induced By Tumor
25 Necrosis Factor And Lymphotoxin," Amer. Jour. Path.,
134, 551 (1989); R. Cotran et al., "Endothelial
Activation Its Role In Inflammatory And Immune
Reactions," in Endothelial Cell Biology, (Plenum Press,
Simonescu & Simonescu, eds., 1988) 335]. TNF also
30 appears to be involved in T cell activation under
certain conditions. [M. Shalaby et al., "The
Involvement Of Human Tumor Necrosis Factors-α And-β In
The Mixed Lymphocyte Reaction," J. Immunol., 141, 499
(1988); N. Damle et al., "Distinct Regulatory Effects
35 of IL-4 and TNF-α During CD3-Dependent and CD3-

Independent Initiation Of Human T-Cell Activation,"
Lymph. Res., 8, 85 (1989); G. Ranges et al., "Tumor
Necrosis Factor-α As A Proliferative Signal For An IL-
2-Dependent T Cell Line: Strict Species Specificity of
5 Action," Amer. Assoc. Immunol., 142, 1203 (1989);
G. Ranges et al., "Tumor Necrosis Factor α/Cachectin Is
A Growth Factor For Thymocytes," J. Exp. Med., 167,
1472 (1988); P. Scheurich et al., "Immunoregulatory
Activity Of Recombinant Human Tumor Necrosis Factor
10 (TNF)-α: Induction Of TNF Receptors On Human T Cells
And TNF-α-Mediated Enhancement Of T Cell Responses,"
J. Immunol., 138, 1786 (1987)].

TNF is produced by several types of cells,
including monocytes, fibroblasts, T cells and Natural
15 Killer (NK) cells. [D. Goeddel et al., "Tumor Necrosis
Factors: Gene Structure And Biological Activities,"
Cold Spring Harbor Symposium Quant. Biol., 51, 597
(1986); D. Spriggs et al., "Tumor Necrosis Factor
Expression In Human Epithelial Tumor Cell Lines,"
20 J. Clin. Invest., 81, 455 (1988); M. Turner et al.,
"Human T cells From Autoimmune and Normal Individuals
Can Produce Tumor Necrosis Factor," Eur. J. Immunol.,
17, 1807 (1987)]. Investigators have also detected
murine and human forms of TNF that are associated with
25 the surface of various cells either as a transmembrane
protein or a receptor-bound molecule. [B. Luettig
et al., "Evidence For the Existence of Two Forms of
Membrane Tumor Necrosis Factor: An Integral Protein
and a Molecule Attached To Its Receptor," J. Immunol.,
30 143, 4034 (1989); M. Kriegler et al., "A Novel Form of
TNF/Cachectin Is a Cell Surface Cytotoxic Transmembrane
Protein: Ramifications For the Complex Physiology of
TNF," Cell, 53, pp. 45-53 (1988); and M. Kinkhabwala
et al., "A Novel Addition To the T Cell Repertory,"
35 J. Exp. Med., 171, pp. 941-946 (1990)].

LT-α also has many activities, generally similar, but not identical to those of TNF, including tumor necrosis, induction of an antiviral state, activation of polymorphonuclear leukocytes, induction
5 of class I major histocompatibility complex antigens on endothelial cells, induction of adhesion molecules on endothelium and growth hormone stimulation. [N. Ruddle and R. Homer, "The Role of Lymphotoxin in Inflammation," Prog. Allergy, 40, pp. 162-182 (1988)].
10 Both LT-α and TNF are ligands to members of the nerve growth factor (NGF) receptor family. [S. Mallett and A.N. Barclay, "A New Superfamily Of Cell Surface Proteins Related To The Nerve Growth Factor Receptor," Immunology Today, 12, 7, 220-223 (1991).]
15   In contrast to TNF, LT-α secretion appears to be a specific property of only activated T cells and certain B-lymphoblastoid tumors. [N. Paul et al., "Lymphotoxin," Ann. Rev. Immunol., 6, 407 (1988)]. Some researchers have also indicated that a membrane-
20 associated form of LT-α may be expressed on the surface of lymphocytes under certain circumstances [J. Hiserodt, et al., "Identification of Membrane-Associated Lymphotoxin (LT) On Mitogen-Activated Human Lymphocytes Using Heterologous Anti-LT Antisera In
25 Vitro," Cell. Immunol., 34, pp. 326-339 (1977); C. Ware et al., "Mechanisms of Lymphocyte-Mediated Cytotoxicity," J. Immunol., 126, pp. 1927-1933 (1981); U. Anderson et al. J. Immunol. Methods, 123, 233 (1989); Y. Abe et al., Jpn. J. Canc. Res., 82, 23
30 (1991); Y. Abe et al., "Studies of Membrane Associated and Soluble (Secreted) Lymphotoxin In Human Lymphokine-Activated T-Killer Cells In Vitro," Lymphokine and Cytokine Research, 11, 2, 115-121 (1992)].
        In recent years genes for both TNF and LT-α
35 have been isolated and cloned, leading to their

complete characterization and to the availability of recombinant forms of both proteins. [P. Gray et al., "Cloning and Expression of cDNA For Human Lymphotoxin, A Lymphokine With Tumor Necrosis Activity," Nature,

5   312, pp. 121-124 (1984); D. Pennica et al., "Human Tumor Necrosis Factor: Precusor Structure, Expression And Homology To Lymphotoxin," Nature, 312, 724 (1984)].

Other "cytokine-like" cell surface proteins including the CD40 protein have recently been shown to

10   share certain similarities with TNF and LT-α. Like TNF and LT-α, the CD40 protein is a ligand to members of the TNF/nerve growth factor (NGF) receptor family. [S. Mallett and N. Barclay, Immunology Today, 12, pp. 220-223 (1991)]. The CD40 protein is a 277-amino

15   acid protein expressed on the surface of B lymphocytes, epithelial cells, and some carcinoma cell lines. [R. Armitage et al., Nature, 357, pp. 80-82 (1992); T. Farrah and C. Smith, "Emerging Cytokine Family," Nature, 358, p. 26 (1992)].

20   We have now identified a novel surface protein, lymphotoxin-β (LT-β) or p33. LT-β has been identified on the surface of several types of lymphocyte cells, including OKT3-stimulated primary T cells, antigen specific IL-2 dependent CTL clones, and

25   a PMA-stimulated human T cell hybridoma II-23.D7. LT-β targets LT-α produced in the cell to the cell membrane where LT-β and LT-α appear as a complex (designated "LT-α/LT-β" throughout this disclosure). The LT-α/LT-β complex is believed to be a novel mechanism for

30   membrane expression of LT-α by activated T-cells. [Androlewicz et al., "Lymphotoxin Is Expressed As a Heteromeric complex With A Distinct 33 kDa Glycoprotein On The Surface Of An Activated Human T Cell Hybridoma," Journal Of Biological Chemistry, 267, pp. 2542-2547

35   (1992)]. The LT-α/LT-β complex may exhibit cytolytic

and cell regulatory activity similar to the soluble
LT-α, TNF and CD40 proteins. The membrane-associated
LT-ß complexed with LT-α may represent, as a complex, a
novel ligand for T cell interactions with other cells
5   and may also be useful in targeted cell lysis.

SUMMARY OF THE INVENTION

The novel protein of the present invention
has been named lymphotoxin-ß (LT-ß). This protein is
found on the surface of several types of lymphocyte
10   cells, including OKT3-stimulated primary T cells,
antigen-specific IL-2 dependent CTL clones, and a PMA-
stimulated human T cell hybridoma, II-23.D7. It forms
a novel complex with LT-α and forms complexes with
other LT-ß subunits (e.g., (LT-ß)$_2$ LT-α complexes).
15   LT-ß has a molecular weight of 31-35 kD as
determined by immunoprecipitation and SDS-PAGE. LT-ß
exhibits N-linked glycosylation. The amino acid
sequence of lymphotoxin-ß is set forth in SEQ ID NO:2,
and the amino acid sequences of several soluble
20   lymphotoxin-ß peptides are set forth in SEQ ID NO:4,
SEQ ID NO:6 and SEQ ID NO:8. The DNA sequence coding
for lymphotoxin-ß is set forth in SEQ ID NO:1 and DNA
sequences coding for several soluble lymphotoxin-ß
peptides are set forth in SEQ ID NO:3, SEQ ID NO:5, and
25   SEQ ID NO:7.
LT-ß as a cell membrane protein binds LT-α
during synthesis, thus "targeting" the LT-α to the cell
membrane. In the absence of LT-ß, LT-α is secreted
into the extracellular medium. The LT-α/LT-ß complex
30   is recognized by polyclonal antisera raised against
recombinant lymphotoxin-α (rLT-α) expressed in CHO
cells or by monoclonal antibodies (mAbs) raised against
natural LT-α. Furthermore, antisera that recognize the
LT-α/LT-ß complex and (LT-α)$_3$ block the mixed

lymphocyte reaction (MLR), a standard immunological
assay of the expected proliferative response of T
lymphocytes to allogenic stimulation, i.e., the
introduction of T lymphocytes from another individual,
5   which are recognized as foreign (non-self) by the
"responder" lymphocytes. [See, e.g., M. Shalaby
et al., J. Immunol., 141, 499 (1988)].

The LT-ß protein was purified by affinity
chromatography, partially sequenced and a specific
10   oligonucleotide probe was designed. The cDNA encoding
the LT-ß was isolated by probing a cDNA library from
activated II-23.D7 cells, a human T-cell hybridoma that
displays large amounts of surface lymphotoxin upon
phorbol ester activation. The identified amino acid
15   sequence encodes a 240-244 amino acid sequence (a
molecular mass of the unmodified protein of about
25130-25390 kDa). See SEQ ID NO:2. The amino acid
sequence and the placement of the transmembrane region
are typical of a type II membrane protein.
20   This sequence comprises a short 14-18 amino
acid N-terminal "cytoplasmic" domain. Following this
cytoplasmic domain there is an extensive stretch of 30
hydrophobic amino acids which presumably acts as a
membrane anchoring domain. No identical sequences were
25   found within available databases. There is one
cysteine residue in the extracellular domain and two
methionines within the last C-terminal 17 amino acids.
This is consistent with the very limited cyanogen
bromide cleavage pattern exhibited by this protein.
30   Comparison of the LT-ß sequence with other
proteins known to bind to members of the TNF/NGF
receptor family reveals considerable structural
similarity. Four of the ligands to members of the
TNF/NGF receptor family (TNF, LT-α, LT-ß and the CD40
35   ligand) resemble type II membrane proteins and share at

least four regions of sequence conservation in the extracellular domain as indicated in Figure 14. The conserved TNF and LT-α domains shared with LT-β are likely to be involved in intersubunit interactions and

5   β sheet organization. These regions of conservation can account for the association between LT-α and LT-β. The existence of these homology regions may facilitate engineering the polypeptide to form complexes with, for example, TNF or the CD40 ligand. Such a molecule would

10 have mixed functions and could possibly be used as a custom designed drug. [See J. Fuh et al., "Rational Design Of Potent Antagonists To The Human Growth Hormone Receptor," Science, 256, 1677 (1992)].

We believe that the polypeptide complexes of

15 this invention are important in T cell activation events and are useful in compositions and methods for T cell activation or T cell suppression and as therapeutic agents in the treatment of inflammation or applications requiring cytolytic activities, such as

20 inhibition of tumor cell or neoplasia growth. We also believe that the polypeptide complexes may be important in cellular immunotherapies, including enhancing the tumoricidal properties of tumor infiltrating lymphocytes in Tumor Infiltrating Lymphocyte ("TIL")

25 therapy. TIL immunotherapy may be improved by gene transfer techniques. For example, a gene may be added to tumor cells for the purpose of inducing the body's immune system to mediate an effective tumor-directed immune response. [See, e.g., W.F. Anderson, "Human

30 Gene Therapy." Science, 256, 808-813 (1992)].

We also believe, based upon similarities between a molecule identified as "Fas" and members of the TNF/NGF receptor family, that the polypeptide complexes of this invention may be involved in the

35 internal cell process known as programmed cell death or

apoptosis, and may therefore be involved in mediating
autoimmune disease. [See, e.g., N. Itoh et al., "The
Polypeptide Encoded By The cDNA For Human Cell Surface
Antigen Fas Can Mediate Apoptosis," Cell, 66, 233-243
5   (1991); R. Watanabe-Fukunaga et al.,
"Lymphoproliferation Disorder In Mice Explained By
Defects In Fas Antigen That Mediates Apoptosis," Nature
356, 314 (1992)].

Antibodies to LT-ß, its related polypeptides,
10   the LT-α/LT-ß complex or the other polypeptide
complexes of this invention may also disrupt critical
LT interactions with particular receptors, thus
specifically affecting LT-mediated events other than
those mediated through the known TNF receptor forms.
15   Likewise, receptors for TNF, LT-α or LT-α/LT-ß, or
their derivatives (e.g., soluble receptors and
IgG/receptor fusion proteins) may be used to inhibit
the polypeptides and complexes of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

20   Figure 1 depicts flow cytofluorometric
analysis of OKT3-stimulated, IL-2 expanded peripheral
blood lymphocytes (PBL) showing reaction with 3
different rabbit anti-rLT-α antisera and showing
essentially no reaction with rabbit anti-rTNF antisera.
25   Figure 2 depicts flow cytofluorometric
analysis of a human T cell hybridoma, II-23.D7, showing
the presence (following PMA treatment) of a LT-α-
related epitope on the cell surface.

Figure 3 depicts the ability of PMA activated
30   II-23.D7 cells to bind anti-rLT-α antibodies. Samples
of anti-rLT-α antisera were incubated with PMA-treated
U937 (non-LT-producing) cells, (-O-), PMA-activated II-
23.D7 hybridoma cells (10^8, - - ; 10^7, -·-), and no
cells (control, - -). Serial dilutions of the cell-

free antisera after incubation were added to rLT-α and used in a cytotoxicity assay against L929 (LT-α-sensitive) cells. The plots indicate that rLT-α neutralizing antibodies were removed from the antisera

5 by the activated II-23.D7 cells. .

Figure 4 shows two autoradiographs (A and B) depicting immunoprecipitation of ^{125}I-labeled surface proteins from PMA-activated II-23.D7 cells. Figure 4A shows immunoprecipitation of an approximately 25 kD

10 surface protein (LT-α) and an approximately 33 kD surface protein (p33, or LT-β) by post-immune rabbit anti-rLT-α antiserum but not by pre-immune rabbit serum. Figure 4B shows a 1-dimensional CNBr cleavage map of the 25 kD and 33 kD bands from figure 4A,

15 compared against recombinant TNF (rTNF) produced in E.coli, and recombinant lymphotoxin-α (rLT-α) produced in CHO cells, [J. Browning et al., J. Immunol., 143, 1859 (1989)] both with (+) and without (-) CNBr cleavage.

20 Figure 5 presents autoradiographs showing immunoprecipitation of TNF- and LT-α-related proteins from PMA-stimulated II-23.D7 cells metabolically labeled with ^{35}S-methionine or ^{35}S-cysteine. The figure shows recognition by anti-rLT-α antisera (L), but not

25 preimmune (P) or anti-rTNF antisera (T), of an approximately 25 kD methionine-containing surface protein (LT-α) and an approximately 33 kD methionine-and cysteine-containing surface protein (p33, or LT-β). The autoradiographs also indicate that activated II-

30 23.D7 cells also produce a 26 kD form of TNF and secrete soluble lymphotoxin-α.

Figure 6 depicts affinity purification 1-D CNBr peptide mapping of those proteins from PMA-activated II-23.D7 cells recognized by anti-rLT-α

35 serum. Figure 6A represents SDS PAGE analysis of the

proteins eluted from an anti-LT-α affinity column
prepared from either pre-immune (PRE) or post-immune
(POST) rabbit sera. Figure 6A shows the -33kD and
-20kD protein bands did not bind to an affinity column

5   prepared using preimmune serum (PRE) but did bind to an
affinity column prepared using anti-rLT-α antiserum
(POST). Figure 6B shows partial CNBr cleavage of the
⁻33 kD and ⁻20 kD proteins eluted from the POST column,
compared against rTNF and rLT-α run in parallel. The

10 gels were visualized by silver staining.

Figure 7 presents autoradiographs of the
⁻25 kD and ⁻33 kD ^{125}I-labeled proteins (designated LT-α
and LT-ß, respectively) immunoprecipitated from
activated II-23.D7 cells, treated with N-glycanase (N-

15 gly), with a mixture of neuraminidase and O-glycanase
(O-gly), or with all three enzymes.

Figure 8 depicts the results of a
reimmunoprecipitation of the coprecipitated p33 (LT-ß)
and p25 (LT-α) proteins to further investigate whether

20 they are immunogenically related.

Figure 9 (comprising parts 9A and 9B) shows
the results of isoelectric focusing under denaturing
conditions of the immunoprecipitated p33 (LT-ß) and p25
(LT-α) proteins.

25   Figure 10 (comprising parts 10A and 10B)
shows the results of isoelectric focusing under native
conditions of the immunoprecipitated p33 (LT-ß) and p25
(LT-α) proteins. Together Figs. 9 and 10 indicate that
LT-ß and LT-α form a denaturable complex.

30   Figure 11 depicts flow cytofluorometric
analysis of surface proteins differentially expressed
on T cells and monocytes after stimulation with a
mixture of LPS, IFN-γ and OKT3. From a stimulated PBL
pool, separated T cells were observed to express a

35 surface protein recognized by anti-rLT-α antisera (LT),

whereas separated monocytes expressed a surface protein
recognized by anti-rTNF antisera (TNF).

Figure 12 shows flow cytofluorometric
analysis of surface LT forms on leu-19⁻ and leu-19⁺

5 (i.e., natural killer) cells treated with IL-2.
Analysis of IL-2 treated PBL with both labeled leu-19
and anti-rLT-α confirms that lymphokine-activated
killer (LAK) cells express a surface LT form.

Figure 13 depicts amino acid sequence of LT-β

10 fragments obtained by direct N-terminal sequencing and
in situ trypsin digestion followed by reverse phase
HPLC resolution of the digested peptides.

Figure 14 depicts an amino acid sequence
comparison of four members of the family of ligands

15 binding to members of the TNF/NGF-receptor family
Homology regions are shown in bold type face with
sequence identity indicated with a dot and conserved
sequences with an asterisk. Putative N-linked
glycosylation sites are boxed.

20 Figure 15 depicts northern analysis of LT-α
and LT-β expression. A) Northern blot of several cell
lines showing specific expression of both LT genes in
only Hut-78 and II-23.D7 cells. B) Time course of PMA
induction of LT-α and LT-β mRNAs in II-23.D7 cells.

25 C) Similar analysis of human peripheral blood
lymphocytes activated with either anti-CD3 or IL-2
alone.

Figure 16 depicts expression of LT-α and LT-β
in CHO cells. A) FACS analysis of CHO cells

30 transiently transfected with the LT-β cDNA. Either
parental dHFR-CHO or LT-α expressing CHO cells were
electroporated with either pCDM8 containing an
irrelevant insert (clone 4) or the pCDM8/LT-β plasmid
and stained with control IgG(---) or anti LT-α

35 monoclonal antibodies (___). B) Panel B depicts

expression of LT-α and LT-ß in COS cells. Cells were
transfected with control DNA or the pCDM8/LTß plasmid,
either with or without pCDM8/LTα and stained as per
panel A.

5   DETAILED DESCRIPTION OF THE INVENTION

        In order that the invention herein described
may be fully understood, the following detailed
description is set forth.
        This invention relates to lymphotoxin-ß, a
10   lymphocyte membrane-type polypeptide. The amino acid
sequence of lymphotoxin-ß is set forth in SEQ ID NO:2.
        This polypeptide, also referred to as p33,
has a molecular weight of 31 to 35 kD. The
polypeptides of this invention may be associated with a
15   cell surface or not associated with such a surface.
        This invention also relates to soluble forms
of lymphotoxin-ß. Soluble lymphotoxin-ß peptides are
defined by the amino acid sequence of lymphotoxin-ß
wherein the sequence is cleaved at any point between
20   the end of the transmembrane region (i.e., at about
amino acid #44) and the first homology region at about
amino acid #95. Amino acid sequences of two soluble
lymphotoxin-ß peptides are defined by SEQ ID NO:4 and
SEQ ID NO:6. Several additional soluble lymphotoxin-ß
25   polypeptides comprise the amino acid sequence as
defined by SEQ ID NO:6 with additional amino acid
residues at the 5' end. The additional residues may
comprise the 52 amino acid residues as defined by SEQ
ID NO:8. The soluble lymphotoxin may also comprise an
30   amino acid sequence defined by SEQ ID NO:6 plus a
portion of SEQ ID NO:8 comprising 1 to 51 of the amino
acid residues beginning from the 3' end. Such soluble
peptides may include any number of well known leader
sequences at the 5' end. Such a sequence would allow

the peptides to be expressed in a eukaryotic system.
[See, e.g., Ernst et al. U.S. Patent No. 5,082,783
(1992)].

5 The polypeptide complexes of this invention
comprise a first polypeptide comprising an amino acid
sequence selected from the group of SEQ ID NO:2, SEQ ID
NO:4, SEQ ID NO:6, and the sequence defined by SEQ ID
NO:6 plus the entirety or a portion of SEQ ID NO:8 as
described above, complexed with a second polypeptide
10 selected from the same group and/or with a second
polypeptide such as: lymphotoxin-α, native human or
animal lymphotoxin-α, recombinant lymphotoxin-α,
soluble lymphotoxin-α, secreted lymphotoxin-α, or
lymphotoxin-α-active fragments of any of the above.
15 The novel LT-β peptide forms a complex with
LT-α and forms complexes with other LT-β subunits
(e.g., (LT-β)$_2$ LT-α complexes). These complexes may be
cell associated or not associated with a cell, and may
complex with other type II membrane proteins sharing
20 common homology regions as described above.
The polypeptide complexes are recognized by
polyspecific antisera that recognize recombinant LT-α,
suggesting that the complex exhibits LT-α epitopes.
These antisera include a commercial anti-LT-α
25 monoclonal antibody (Boehringer Mannheim), and
polyspecific antisera raised against recombinant
lymphotoxin-α (rLT-α) expressed in transfected Chinese
Hamster Ovary (CHO) cells. Polyclonal antisera that
recognize these complexes also block the mixed
30 lymphocyte reaction (MLR), but a monoclonal anti-rLT-α
antibody that recognizes soluble LT-α do not block the
MLR. The complexes of the present invention thus
appear to play an important role in T cell activation.
We also expect these complexes to have T cell

regulatory activities and cytotoxic activities similar to those of soluble LT-α or TNF.

This invention also relates to DNA consisting essentially of DNA sequences that code for the
5 polypeptides comprising the amino acid sequences described above, recombinant DNA molecules characterized by that DNA, hosts selected from the group of unicellular hosts or animal and human cells in culture transfected with that DNA, and recombinant
10 methods of using that DNA and those recombinant DNA molecules and hosts to produce the polypeptides coded thereby.

More specifically, this invention relates to an isolated DNA sequence comprising the nucleotide
15 sequence as defined by SEQ ID NO:1.

This invention also relates to polypeptides encoded by that sequence, DNA sequences that hybridize with that DNA sequence that code for a polypeptide that is substantially homologous with lymphotoxin-ß, and
20 degenerate DNA sequences comprising nucleotide sequences that code for lymphotoxin-ß.

This invention also relates to DNA sequences that code for soluble lymphotoxin-ß peptides. These DNA sequences are defined by SEQ ID NO:3 and SEQ ID
25 NO:5. This invention also relates to several additional soluble lymphotoxin-ß peptides that are coded for by the DNA of SEQ ID NO:5 plus several additional nucleotide triplets at the 5' end. The additional nucleotide triplets may comprise the 52
30 triplets as defined by SEQ ID NO:7. The soluble lymphotoxin peptide may also be encoded by SEQ ID NO:5 plus a portion of SEQ ID NO:7 comprising 1 to 51 nucleotide triplets beginning from the 3' end.

This invention also relates to DNA sequences
35 that hybridize to any of the sequences identified above

that code on expression for lymphotoxin-ß or soluble
lymphotoxin-ß peptides. This invention also relates to
degenerate nucleotide sequences that code for
lymphotoxin-ß or a soluble lymphotoxin-ß peptide, and
5   to DNA sequences that code for polypeptides that are
substantially homologous with a soluble lymphotoxin-ß
peptide.

Lymphotoxin-ß was identified, isolated and
characterized using the techniques described below:

10   **Flow Cytofluorometric Analysis**

First we demonstrated the expression of LT-α
epitopes on the surface of T cells using flow
cytofluorometric analysis. We observed that human
peripheral blood mononuclear cells activated with OKT3
15   monoclonal antibody demonstrated expression of LT-α
epitopes by reacting with anti-rLT-α antisera. Only
anti-rLT-α antisera, not anti-rTNF antisera, bound to
OKT3-stimulated primary T cells.

We also observed that a human T cell
20   hybridoma, II-23.D7 [C. Ware et al., "Human T Cell
Hybridomas Producing Cytotoxic Lymphokines: Induction
of Lymphotoxin Release And Killer Cell Activity By
Anti-CD3 Monoclonal Antibody Or Lectins And Phorbol
Ester," Lymph. Res., 5, 313 (1986)], secreted LT-α upon
25   PMA stimulation and also expressed surface LT-α-related
epitopes upon PMA stimulation. We also demonstrated
that PMA-activated II-23.D7 cells were able to remove
LT-neutralizing antibodies from the rabbit anti-rLT-α
antisera, while control U937 cells, which lack all
30   surface LT-α forms, were not. We further ruled out the
possibility that the rabbit anti-rLT-α antisera had
bound (complexed) with rabbit LT-α (the resulting
complexes subsequently binding to cellular LT-α/TNF
receptors on the II-23.D7 cells) by saturating the

cellular receptors with excess soluble TNF or LT-α and observing that this had no effect on the staining. These assays demonstrate that the LT-α-related epitopes on this hybridoma are genuinely related to LT-α.

5   We also observed that pretreatment of the antisera with excess rLT-α blocked the ability of the antisera to stain II-23.D7 cells, while pretreatment with rTNF had no effect. This assay demonstrated the specificity of the antisera for LT-α-related epitopes.

10   Trypsinization of the stimulated II-23.D7 cells prior to staining led to loss of the signal, demonstrating that the epitopes recognized by the antisera were proteins.

        We also demonstrated that CHO-derived

15 contaminants did not contribute to the antisera recognition of induced proteins on the surface of activated II-23.D7 cells by showing that CHO cells stably transfected with the LT-α gene, which produce only soluble LT-α, were not stained by the anti-LT-α

20 antisera.

Immunoprecipitation

        We further characterized these surface LT-α-related proteins by either surface iodination (^{125}I-labelling) or metabolic labelling (^{35}S-Met or ^{35}S-

25 Cys) of PMA-activated II-23.D7 cells, followed by solubilization of the plasma membrane with detergent and immunoprecipitation of the labeled LT-α-related proteins.

        Surface iodination coupled with

30 immunoprecipitation revealed two proteins recognized by the anti-LT-α antisera: a 25-26 kD form subsequently referred to as LT-α, and a 31-35 kD form subsequently referred to as LT-β or p33. We observed that neither the preimmune serum from the same rabbit nor anti-rTNF

rabbit serum immunoprecipitated these bands from the
iodinated, PMA-activated II-23.D7 cells. One
dimensional partial CNBr peptide mapping of the
iodinated, immunoprecipitated bands showed that the
5   25-26 kD form (LT-α) cleaves in a pattern identical to
that of iodinated recombinant LT-α, reinforcing the
correlation between surface LT-α and soluble LT-α. The
iodinated 31-35 kD form (LT-β, or p33) was not cleaved
by CNBr, indicating that it is distinct from the known
10 LT-α gene product.

     We further characterized the LT-α and LT-β
proteins by metabolic labelling of PMA-activated
II-23.D7 cells with ^{35}S-methionine or ^{35}S-cysteine,
followed by immunoprecipitation. The distribution of
15 cysteine and methionine provides a means of
distinguishing between TNF and LT-α and between forms
of each with and without their signal sequences
[M. Kriegler et al., Cell, 53, 45 (1988)]. Secreted
TNF contains cysteine, but not methionine, while
20 secreted LT-α contains only methionine and no cysteine
residues. LT-α, however, has one cysteine residue in
its signal sequence, while TNF contains two methionine
residues in its signal sequence.

     We labeled separate cultures of PMA-treated
25 II-23.D7 cells with either ^{35}S-methionine or
^{35}S-cysteine and precipitated immunoreactive proteins
from the culture media and the cells. Subsequent SDS-
PAGE analysis of the immunoprecipitates from the
culture media of cells labeled with ^{35}S-methionine
30 revealed a 25 kD form of LT-α while the
immunoprecipitates from the culture media of cells
labeled with ^{35}S-cysteine did not, a pattern expected
for secreted LT-α. Analysis of the washed cells showed
both the 25-26 kD LT-α form and the 33 kD LT-β form.

These results parallelled the membrane-associated forms observed using surface iodination.

The 25-26 kD LT-α lacked cysteine, indicating processing of the leader sequence. We also observed

5 that the 33 kD LT-ß incorporated both ^{35}S-methionine and ^{35}S-cysteine, distinguishing itself as different from the 25 kD LT-α form. Typically, LT-α bound to its receptor can be cross-linked to the receptor using a chemical linker such as BOSCOES (i.e., (bis

10 [2-[succinimidooxy-carbonyloxy]ethyl] sulfone; Pierce, Rockford, IL). [J.S. Andrews et al., "Characterization of the Receptor for Tumor-Necrosis Factor (TNF) and Lymphotoxin (LT) on Human T Lymphocytes," J. Immunol., 144, 2582 (1990)]. We observed that when surface

15 iodinated II-23.D7 cells were treated with a cross-linking agent, there was no association of either the 25-26 kD LT-α or the 33 kD LT-ß related form with an additional membrane protein. This assay demonstrated that receptor binding is not the mechanism by which

20 LT-α and LT-ß remain associated with the cell membrane. Sequence analysis of LT-ß showed no relationship to either of the two TNF-receptor forms [C. Smith et al., Science, 248, 1019 (1990); T. J. Schall et al., Cell, 61, 361 (1990)].

25 Affinity Chromatography

Further characterization of the LT-ß and LT-α proteins on the surface of II-23.D7 cells was obtained through affinity chromatography. We observed that LT-α/LT-ß complex on the surface of PMA-treated II-

30 23.D7 cells bound to lentil lectin, indicating a glycoprotein structure for each form. Hence a lentil lectin chromatography step was used as a purification step prior to antisera affinity chromatography. We bound detergent-solubilized PMA-treated II-23.D7

proteins to lentil lectin sepharose and eluted with α-
methyl mannoside. We prepared both control IgG and
anti-LT-α-IgG affinity columns to accurately assess
those proteins specifically recognized by the anti-

5   LT-α antiserum. We then applied the proteins that
bound to lentil lectin to these columns. We observed
that low pH elution of the columns led to the release
of the LT-ß and LT-α proteins from the anti-LT-α
affinity column. SDS-PAGE analysis of the eluate

10   closely resembled the SDS-PAGE analysis of
immunoprecipitated proteins from surface iodinated PMA-
treated II-23.D7 cells. This comparison demonstrated
that similar proteins were purified by the two methods.
We observed that during affinity

15   purification, the ⁻25 kD LT-α form appeared to be
cleaved to a 19-20 kD form, or a "des 20" form. The
original isolation of natural LT-α from the RPMI 1788
tumor cell line [B. Aggarwal et al., "Primary Structure
of Human Lymphotoxin Derived From 1788 Lymphoblastoid

20   Cell Line," J. Biol. Chem., 260, pp. 2334-2344 (1985)]
also yielded an N-terminally cleaved 20 kD LT-α form.
One of the methionines is lost in this "des-20" natural
LT-α form, producing a different CNBr cleavage pattern
from the intact molecule. One-dimensional CNBr digests

25   of the affinity-purified LT-α protein demonstrated a
cleavage pattern that is consistent with the truncated
natural LT-α form, and we concluded that the affinity-
purified "des-20" LT-α form probably results from a
similar cleavage as observed with the natural LT-α

30   "des-20" form.

We further observed that LT-ß generates a
doublet upon partial CNBr cleavage. The cleavage
pattern generated by the LT-ß protein demonstrated that
methionine residue(s) were present, and at least one

35   methionine was within 5-20 residues from either the

C-or N-terminus (methionine residues within 1-5
residues of either end would not be detected when
cleaved using this mapping technique). This pattern
suggested that LT-ß does not contain the entire known
5   LT-α sequence.

We observed that the LT-ß protein is also
expressed by antigen-activated primary cytotoxic T
lymphocyte clones. Metabolic labelling of these cells
followed by immunoprecipitation with anti-rLT-α
10 revealed LT-ß along with small amounts of LT-α. These
results demonstrated that LT-ß is made by primary T
cells as well as by the II-23.D7 hybridoma.

<u>Initial purification of the LT-ß and LT-α proteins</u>

We purified these LT-α and LT-ß proteins
15 using the following general steps. We first added
phorbol myristic acetate (PMA) to II-23.D7 cells.
After 24 hours we harvested the cells and washed them
with cold serum-free RPMI medium. To the chilled cell
pellet we added ice-cold lysis buffer (HEPES, NP-40,
20 EDTA, NaCl, and sodium azide) to which benzamidine,
phenyl methyl sulfonyl chloride (PMSF), and N-ethyl
maleimide (NEM), soybean trypsin inhibitor, pepstatin
and aprotinin had been freshly added. We homogenized
the cells gently in a Dounce homogenizer and
25 centrifuged the lysate. We centrifuged and collected
the supernatant. We loaded the supernatant onto a
lentil-lectin sepharose column equilibrated in lysis
buffer to which we had added $CaCl_2$ and $MnCl_2$. We
washed the column with lysis buffer with $CaCl_2$ and
30 $MnCl_2$ and then eluted with lysis buffer containing α-
methyl mannoside. We pooled the eluate fractions and
loaded directly onto a rabbit nonspecific IgG sepharose
affinity column which was directly connected to a
rabbit anti-rLT-α sepharose affinity column. We washed

both columns with the same lysis buffer with EDTA
followed by lysis buffer wherein the NP-40 had been
replaced with MEGA-8 (Octanoyl-N-methyl glucamide,
Boehringer-Mannheim). We eluted the washed columns
5   individually with a solution of 5mM MEGA-8, 50mM
glycine, NaCl, benzamidine, and EDTA as described in
Browning et al., "Lymphotoxin And An Associated 33-kDa
Glycoprotein are Expressed On The Surface Of An
Activated Human T-Cell Hybridoma," J. Immunol. 147,
10   pp. 1230-1237 (1991). The first fractions following
the pH shift were pooled, lyophilized and resuspended
in water with SDS, and dialyzed against a solution of .
HEPES and SDS. We dried the dialyzed fractions on a
speed-vac and resuspended in water. We mixed aliquots
15   with Laemmli loading buffer and electrophoresed on SDS-
PAGE. We visualized proteins by silver staining.
        We observed that LT-α epitope(s) are present
on the surface of the II-23.D7 T cell hybridoma only
following cell activation such as occurs with PMA
20   treatment. In contrast, when present on primary
T-cells, PMA treatment leads to loss of the surface
antigen. Additionally, we found that rabbit polyclonal
antisera to either recombinant LT-α (produced in CHO
cells) or natural LT-α (e.g., Genzyme, Boston, Mass.)
25   recognized the LT-α epitope(s).
        We have also observed that our antisera
recognizing the LT-α/LT-β complex blocks the MLR,
whereas a particular monoclonal antibody recognizing a
soluble LT-α does not [M. Shalaby et al., J. Immunol.,
30   141, 499 (1988)]. The LT-α/LT-β complex of the
invention, therefore, may be a mediator in T cell
activation.
        The presence of LT-β with LT-α in
immunoprecipitates from cell lysates suggested that
35   either LT-β is antigenically related to LT-α or that

LT-ß is bound to LT-α or both. To address this issue 25 kD and 33 kD bands from ^{35}S-methionine labeled cells were immunoprecipitated with rabbit polyclonal anti-rLT-α serum, eluted from excised gel slices and

5   subjected to reimmunoprecipitation with either anti-rLT-α polyclonal serum or anti-rLT-α mAb. LT-α, but not LT-ß, could be immunoprecipitated with either of the anti-rLT-α antibodies, suggesting that LT-ß is not antigenically related to LT-α. These observations

10   indicated that LT-ß is physically associated with LT-α.

        To further investigate the hypothesis that surface LT-α and LT-ß form a complex, we performed isoelectric focusing (IEF) experiments under both denaturing and native conditions, the rationale being

15   that if LT-α and LT-ß are physically associated, then they should focus as a complex under native conditions but as separate entities under denaturing conditions. The individual isoelectric points (pI's) for LT-α and LT-ß were determined by two-dimensional gel analysis

20   (denaturing conditions) (Fig. 9A). LT-α possesses five charged isomers ranging in pI from 6.5 to 7.3, whereas LT-ß possesses four charged isomers ranging in pI from 5.5 to 6.0. When focusing was performed under native conditions, however, LT-α and LT-ß focused together as

25   a broad band ranging in pI from 6.3 to 7.2 (Fig. 10A, lanes 6-8). Therefore, the migration of LT-ß was significantly retarded under native conditions.

Further purification and identification of LT-α and LT-ß

30   We later purified these LT-α and LT-ß proteins using the following general steps. We grew II-23.D7 cells in RPMI medium with fetal bovine serum and we harvested the cells from 50 l of RPMI and resuspended them in medium and we added phorbol

35   myristoyl acetate (PMA). After activation for 6 hours,

we harvested the cells by centrifugation and washed
them with Dulbecco's phosphate buffered saline. We
suspended the final cell pellet in cold lysis buffer
and passed the pellet once through a nitrogen
5   cavitator. We centrifuged the lysed cells and
discarded the supernatant. We extracted the pellet
overnight in lysis buffer with detergent and then
centrifuged it again.

      We added the supernatant containing the
10 detergent solubilized membranes to affinity resin
composed of monoclonal anti-LT-α coupled to Affi-gel
(10) and rocked the suspension overnight. We collected
the resin into a small column and washed it with HEPES
with nonidet P40, and then with the same buffer with 1%
15 w/v MEGA-8, we eluted the bound proteins with MEGA-8 in
glycine buffer and neutralized the fractions
immediately with Tris base. We determined the presence
of LT-β and LT-α in the fractions by SDS-PAGE analysis
and silver staining. We pooled fractions containing
20 these proteins and added SDS, and we dialyzed the pool
against 0.1x laemmli sample buffer (using multiple
changes to remove the MEGA-8 detergent). We
lyophilized the dialyzed solution to dryness and
resuspended it in 1/10th the original volume of water.

25   We ran the sample on an SDS-PAGE gel, blotted
onto a ProBlot membrane and stained with Coomassie blue
dye. We excised the LT-β and LT-α bands and loaded
them into a protein sequencer. We obtained the
N-terminal sequence by Edman degradation. We found the
30 sequence of the membrane associated LT-α band to
exactly match that described for secreted LT-α, i.e.,
Leu Pro Gly Val Gly Leu Thr Pro Ser (amino acid No. 1
to 9.) [P. Gray et al., Nature, 312, pp. 121-124
(1984)]. The Edman degradation analysis revealed that
35 the N-terminal portion of the associated LT-β protein

included two possible amino acid sequences: Gly Leu
Glu Gly Arg Gly Gln Arg Leu Gln or Gly Leu Glu Gly Arg
Leu Gln Arg Leu Gln. Subsequent DNA analysis using
more accurate cDNA techniques confirmed that the
5   correct sequence was Gly Leu Glu Gly Arg Gly Gly Arg
Leu Gln.

In each case where a surface LT-α form was
detected, we were also able to detect LT-β (i.e., in
PMA-activated II-23.D7, activated CTL clones, and Hut-
10 78 cells constitutively expressing a surface LT-α
form). Because LT-α is secreted from transfected CHO
cells in the absence of a surface LT-α form, and
because the presence of LT-β is associated with
surface-bound LT-α, we concluded that LT-β complexes
15 with LT-α to target it to the cell surface.
Biochemically, LT-β and LT-α co-migrate on a non-
denaturing isoelectric focusing gel, but when the
complex is dissociated with urea, the two proteins run
separately. [See Figs. 9A, 10A.] These observations
20 have led us to conclude that LT-α and LT-β exist as a
complex on the cell surface.

Identifying DNA Sequences That Code For
Lymphotoxin-β And Soluble Lymphotoxin-β Peptides

Lymphotoxin-β was purified by immunoaffinity-
25 chromatography as described above. Direct N-terminal
sequencing and in situ trypsin digestion followed by
reverse phase HPLC resolution of the digested peptides
was performed. [See, Abersold et al., "Internal Amino
Acid Sequence Analysis Of Proteins Separated By One Or
30 Two Dimensional Gel Electropherisis After In Situ
Protease Digestion On Nitrocellulose," PNAS, 84, 6970-
6974 (1987)]. The resulting N-terminal and internal
tryptic fragment peptides were then sequenced using
conventional methods. The sequencing of the N-terminus

and internal peptides, designated as T105, T87/88, T110
and T67 are shown in Fig. 13.

Two antisense 17-mer oligonucleotide probes
GTYTCNGGCTCYTCYTC [SEQ ID NO:9] and GTYTCNGGTTCYTCYTC
5 [SEQ ID NO:10], designated 1368 and 1369, respectively,
were synthesized to match a portion the sequence of
peptide T-87/T-88 and radiolabelled with ^{32}P. Northern
analysis showed that the probe designated 1368
hybridized strongly to a 0.9-1.1 kb mRNA band that was
10 strongly induced in II-23.D7 cells that had been
pretreated with phorbol ester as previously described.

A cDNA library in the vector pCDM8 was
constructed from poly A+ mRNA isolated from II-23.D7
cells induced with PMA for 6 hours. The library was
15 screened with the labelled oligomer designated 1368 and
positive clones were isolated following washing with
3 M tetramethylammonium chloride at 50°C. Several
(>16) clones containing 0.8-0.9 kb inserts were
subjected to DNA sequence analysis.
20 Clone pCDM8/LT-ß-12 contained the coding
sequence of lymphotoxin-ß as shown in SEQ ID NO:1. The
other clones were identical except for various
truncations at the 5' end. The clone 12 cDNA codes for
a functional lymphotoxin-ß. Using standard primer
25 extension methods, three additional codons encoding the
amino acid residues -- MET GLY ALA -- were identified.
A termination sequence AATAAA at position 862-867 was
found just prior to the 3' poly A tract indicating that
the entire 3' end had been identified. The protein
30 coding sequence encodes for 240 amino acids with a
calculated unmodified molecular weight of approximately
25,390 kDa.

The 5' end of the LT-ß DNA sequence
identified from the clone 12 cDNA and primer extension,
35 ATGGGGGCACTGGGGCTG [SEQ ID NO:11] reveals three

possible start codons (underlined). [See, e.g.,
M. Kozak, "An Analysis Of Vertebrate mRNA Sequences:
Intimations Of Translational Control," J. Cell. Biol.,
115, 4, 887-903 (1991).] Engineered LT-ß polypeptides

5 and DNA sequences may be derived by cleaving all or
part of this 5' sequence and substituting a single
start codon.

This amino acid sequence profile is typical
of a type II membrane protein. Following a short

10 (maximum 17) amino acid N-terminal "cytoplasmic" domain
there is an extensive stretch of 30 hydrophobic amino
acids which presumably acts as a membrane anchoring
domain. No identical sequences were found within the
available databases. There is one cysteine residue in

15 the extracellular domain and two methionines within the
last C-terminal 17 amino acids. This is consistent
with the very limited cyanogen bromide cleavage pattern
exhibited by this protein.

After the sequence of LT-ß was determined,

20 subsequent comparison revealed that LT-ß is a type-II
membrane protein with significant homology to TNF, LT-α
and the CD40 protein. These polypeptides share four
regions of sequence conservation in the extracellular
domain. See FIG. 14. Such conservation regions are

25 likely to enable those polypeptides to form complexes
with each other. [See, e.g., M. Eck and S. Sprang,
"The Structure Of Tumor Necrosis Factor-α At 2.6Å
Resolution, Implications For Receptor Binding," J.
Biological Chemistry, 264, 29, pp. 17595-17605 (1989);

30 E. Jones et al., "Structure Of Tumor Necrosis Factors,"
Nature, 338, pp. 225-228 (1989); M. Eck et al., "The
Structure of Human Lymphotoxin (Tumor Necrosis
Factor-ß) At 1.9-Å Resolution, J. Biological Chemistry,
267, 4 pp. 2119-2112 (1992); J. Tavernier et al.,·

35 "Conserved Residues Of Tumor Necrosis Factor And

Lymphotoxin Constitute the Framework Of The Trimeric Structure," Fed. Eur. Biochem. Soc. Lett., 257, 2 (1989)].

Expression of Cloned LT-β

5   The pCDM8/LT-β clone 12* or a control plasmid, Clone 4 (pCDM8 with a non-functional LT-β cDNA insert), were introduced by electroporation into CHO dhfr⁻ cells and CHO cells stably transfected with human LT-α. After three days, cells were removed with Ca/Mg-
10   free Hank's solution with 5 mM EDTA and stained for FACS analysis as described above using either 10 μg/ml control IgG1 or anti-LTα monoclonal antibody (Boehringer-Mannheim) followed by labelling of bound immunoglobulin with either a FITC or phycoerythrin
15   labelled goat anti-mouse preparation.

         In other experiments, COS cells were electroporated with either clone 4 or clone 12 LT-β cDNA in pCDM8 in the presence or absence of an equal amount of human LT-α cDNA also in the pCDM8 vector and
20   stained for FACS analysis after three days as above. Only CHO cells expressing LT-α displayed surface lymphotoxin upon transfection with a functional LT-β DNA, i.e., clone 12.

         Clone 12 lacks an initiating ATG codon, but
25   does possess several CTG initiating codons and hence this expression experiment shows that one or several of the 5' CTG codons must initiate translation. CTG codons are known to serve as initiating sites for translation in several eukaryotic proteins [M. Kozak,
30   J. Cell. Biol., 115, 4, 887-903 (1991)]. Similar results were observed using the dual transfection

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*   E.coli K12 bearing a plasmid pCDM8/LT-β clone 12, designated BN1289 (MC1061/P3/P33-clone-12) was deposited with ATCC on November 13, 1992.

system in COS cells, such that only COS cells receiving both LT-α and LT-ß DNA displayed substantial surface LT-α in a FACS analysis.

Potential uses of LT-ß and LT-α and
5 the LT-α/LT-ß complex

As noted above, there is considerable structural similarity between LT-ß, LT-α, TNF and the CD40 ligand. LT-ß, LT-α, TNF and the CD40 ligand are type II membrane proteins and share at least four
10 regions of sequence conservation in the extracellular domain.

In light of this structural similarity, it is of interest that LT-α is found on the surface of activated lymphocytes in a form identical to the
15 secreted molecule but complexed with an additional integral membrane protein presumably anchoring LT-α to the surface. We believe that this unique complex, now determined to be LT-α/LT-ß, represents a more relevant form of LT-α and imparts specificity relative to TNF.
20   The existence of a heterometric complex of lymphokines, while unique to the immune system is reminiscent of signalling molecules in other areas, e.g, the PDGF and inhibin/activin heteromeric complexes. Delineation of the LT-α/LT-ß complex poses
25 the possibility of immunoregulatory activities unique to the complex which cannot be mimicked by the LT-α homotrimer. The complex may bind to a unique receptor or receptor chain combinations leading to a high affinity interaction and biologically relevant
30 signalling. The hypothesis of an LT-α/LT-ß interaction with a unique receptor complex could account for the relatively poor activity of the LT-α homotrimer relative to TNF in many systems, an observation which cannot be explained by studies on the two known TNF

receptors. [T. Schall et al., Cell, 61, 361-370
(1990); C. Smith et al., Science, 248, 1019 (1990)].

    The tethering of soluble LT-α to the cell
surface via complexation with LT-β indicates that cell-
5 cell contact specific signalling through LT-α/LT-β may
be an important aspect of immune regulation. Because
the TNF and related LT-α forms are secreted we believe
that LT-β may also be secreted. This may be verified
in studies using anti-LT-β monoclonal antibodies. Such
10 antibodies may also be used to determine whether LT-β
homo-oligomers occur naturally.

    In general, LT-α and TNF exhibit
qualitatively the same spectra of activities, and LT-α
and TNF are believed to interact with the same set of
15 receptors (designated the 55 and 80 kD TNF receptors).
[C. Smith et al., Science, 248, 1019 (1990); T. Schall
et al., Cell, 61, 361 (1990)]. Nonetheless, the
quantitative patterns of biological potency exhibited
by LT-α and TNF are dramatically different, with LT-α
20 often being much less potent than TNF [see, e.g.,
Browning et al., J. Immunol., 143, 1859 (1989)]. These
observations are difficult to reconcile with the
existing receptor binding data. It is possible that
the LT-α/LT-β complex imparts unique properties on LT-α
25 such that it now interacts with other as yet undefined
receptors. In this case, a LT-α/LT-β complex and the
other complexes of this invention would have unique
biological properties distinguishing them from either
LT-α or TNF. The LT-α/LT-β complex may be used to
30 identify and clone such LT-α/LT-β or LT-β specific
receptors. Moreover, further use of the complex may
reveal novel biological activities.

    Also, while a number of T cell and macrophage
cell lines are known to be infectable by the HIV virus,
35 in practice only a small number of cell lines have been

useful in propagating the virus in tissue culture. For example, the H9 line, a derivative of Hut-78 originally exploited by Gallo et al. [M. Popovic et al., Science, 224, 497-500 (1984)], and another human lymphocytic
5 line, C8166, have been valuable for HIV propagation [M. Somasundaran and H. Robinson, Science, 242, 1554-1557 (1988)]. It is possible that surface LT-α expression or the capacity for expression of surface LT-α makes a given cell a good target for HIV
10 proliferation.

A role for TNF has been proposed in enhancing HIV proliferation [L. Osborn et al., Proc. Natl. Acad. Sci. USA, 86, 2336 (1989); Z. Rosenberg and A. Fauci, Immunol. Today, 11, 176 (1990); C. Locardi et al.,
15 J. Virology, 64, 5874 (1990); G. Oli et al., Proc. Natl. Acad. Sci. USA, 87, 782 (1990)]. We have found that the II-23.D7 line is infectable with the HIV strain IIIB, but upon PMA treatment the infection by the virus is dramatically increased. The Hut-78 cell
20 line was found to constitutively express a surface LT form, and the C8166 line resembles II-23.D7 in that surface LT appears following PMA treatment. [Ware et al., J. Immunol., article in press (1992)].

Considering these results on the
25 infectability of II-23.D7 by HIV and the relationship between infectable cell lines and surface LT expression, we propose that those lines may be good hosts for HIV infection and replication because the LT-α/LT-β complex and the other polypeptides and
30 complexes of this invention serve a regulatory role. It has been demonstrated that the LT-α gene is induced by expression of the HIV transcriptional activator TAT [K. Sastry et al., J. Biol. Chem., 265, 20091 (1990)] and, moreover, HTLV-1 infection has also been shown to
35 induce LT-α expression [N. Paul, et al., J. Virol., 64,

5412 (1990); E. Tschachler et al., in Human
Retrovirology (Raven Press 1990), W. Blattner, eds., p.
105]. Thus, induction of LT-α by HIV infection and
consequent LT-α/LT-ß complex or other complex

5 expression, or induction of LT-α/LT-ß or other complex
expression by PMA treatment in cell lines competent to
make these proteins, may serve to enhance viral
replication. For this reason, antibodies or specific
binding proteins (e.g., soluble receptors) to the

10 LT-α/LT-ß complex or the other polypeptide complexes of
this invention or to soluble forms of those complexes
or to LT-ß and the other polypeptides of this invention
may inhibit HIV proliferation or block HIV-induced T
cell death.

15   Parallels may be drawn between LT-α/LT-ß and
the CD40 receptor ligand pair where signalling from a T
cell surface CD40 ligand provides "help" to the B cell
via the CD40 receptor. One could therefore postulate
that surface LT-α/LT-ß may be a component of T cell

20 regulation of T cells or other cells of hematopoietic
lineage such as LAK or NK cells and B-cells. Moreover,
this interaction may be dysfunctional in some
autoimmune diseases. [See, e.g., R. Watanabe-Fukunaga,
Nature, 356, pp. 314-317 (1992).]

25   Furthermore, a cell surface protein
designated as the Fas antigen has been shown to have
considerable structural homology with a number of cell-
surface receptors including TNF, NGF and the CD40
protein. The Fas antigen has been implicated in

30 mediating apoptosis, a process also referred to as
programmed cell death [R. Watanabe-Fukunaga, Nature,
356, pp. 314-317 (1992); N. Itoh et al., Cell, 66,
pp. 233-243 (1991)]. A strain of mice that
demonstrates defects in the Fas antigen develop a

35 systemic lupus erythematosus-like autoimmune disease.

This suggests that the structurally similar LT-ß or
LT-α/LT-ß complexes may also play a role in mediating
systemic lupus erthyematosus and, therefore,
intervention in this pathway may be clinically useful
5   in treating various autoimmune diseases.
Alternatively, LT-ß or an LT-α/LT-ß complex may be
involved in inducing programmed cell death through a
cell-cell contact dependent mechanism. The emergence
of this family of TNF related ligands to complement the
10 already extensive family of TNF/NGF type receptors
suggests the existence of an additional array of
important regulatory elements within the immune system.

LT-ß or a LT-ß/LT-α complex may similarly
play a role in suppressing the immune system and may be
15 potentially useful in treating allergy and inducing
tolerance.

The location of the TNF/LT locus in the MHC
region of the genome has led workers to examine linkage
to various autoimmune disease, especially
20 insulin-dependent diabetes melitis. [See, e.g.,
F. Pociot et al., "A Tumor Necrosis Factor Beta Gene
Polymorphism In Relation To Monokine Secretion And
Insulin-Dependent Diabetes Mellitus," Scand. J.
Immunol., 33, 37-49 (1991); K. Badenhoop et al., "TNF-
25 α Gene Polymorphisms In type 1 (Insulin-Dependent)
Diabetes Mellitus," Diabetologia, 32, 445-448 (1989).]
Because we found that the LT-ß gene is located next to
the TNF/LT locus, it is possible that the LT-ß gene or
its receptor may be involved in this autoimmune
30 condition. Hence, LT-ß or its receptor or antibodies
to LT-ß may comprise a replacement therapy in this form
of diabetes.

As discussed above, the LT-ß polypeptide, and
the polypeptide complexes of this invention, are ·
35 expected to have a number of potential uses including

anti-tumor, T cell activating, or T cell suppressing applications, application involving the treatment of systemic lupus erythematosus, as well as uses in anti-inflammatory compositions and methods. DNA sequences

5 coding for LT-ß polypeptides, recombinant DNA molecules including such DNA sequences, and unicellular hosts and animal or human cells in culture transfected with such recombinant DNA molecules may then be employed to produce large amounts of the polypeptides of this

10 invention, substantially free from other human proteins, for use in the compositions and therapies noted above.

Lymphocytes expressing on their surfaces the polypeptide complexes of this invention, and preferably

15 an LT-α/LT-ß complex, represent a subset of lymphocytes that may have enhanced abilities to kill tumor cells. As such, this subset would be useful in LAK (lymphokine-activated killer) cell or TIL (Tumor Infiltrating Lymphocyte) cell therapies. [H. Thomas,

20 K. Sikora, "Biological Approaches to Cancer Therapy," Jour. Int. Med. Res., 17, 191 (1989)]. TIL immunotherapy may be improved by gene transfer techniques. Recombinant genes for LT-ß and related polypeptides based thereon will be useful

25 therapeutically, for example in TIL therapy, where a LT-ß gene, either with or without an LT-α gene, is introduced into T cells isolated from a tumor and introduced to the patient. More preferably, the cells are taken from the patient, transfected with a DNA

30 sequence encoding on expression a polypeptide of this invention, before or after that transfection incubated with a lymphokine, preferably IL-2, and returned to the patient. The transfected T cells (now expressing LT-ß and also consequently complexing LT-α) home in on the

35 tumors from which they were removed, where the

tumorcidal action of LT-α is delivered directly to the tumors. Likewise, it is contemplated that a LT-β gene introduced into LAK cells would increase the number of surface complexes on the cells and enhance their

5 activity. Alternatively, introduction of the LT-β gene into a patient's tumor cells may be useful in creating a tumor vaccine in which the LT-β modified tumor would trigger an enhanced immune response to the tumor itself. [See, e.g., W.F. Anderson, Science, 256, 808-

10 813 (1992)].

Antibodies or antibody derivatives to the polypeptides and polypeptide complexes of this invention are also useful in conventional immunological methods, e.g., panning or flow cytofluorometric

15 sorting, to enrich for this cell population. [L.J. Wysocki and V.L. Sato, "Panning for Lymphocytes: A method for Cell Selection," PNAS 75, 2844 (1978)].

It is also contemplated that the polypeptides and polypeptide complexes of this invention, or

20 fragments or derivatives thereof, will be useful in cell regulatory or therapeutic applications similar to those in which lymphotoxin-α and tumor necrosis factors are used.

The compositions of this invention will be

25 administered at an effective dose to treat the particular clinical condition addressed. Determination of the particular dose for a given application is well within the skill of the art taking into consideration, for example, the condition and weight of the patient,

30 the extent of desired treatment and the tolerance of the patient for the treatment. Administration of the complexes and polypeptides of this invention, or perhaps peptides derived or synthesized therefrom or using their amino acid sequences, including isolated

35 and purified forms of the polypeptides, or their salts

or pharmaceutically acceptable derivatives thereof, may
be via any of the conventionally accepted modes of
administration of agents which exhibit anti-tumor, T
cell-activating, T cell-suppressing or anti-

5   inflammatory activity.

The compositions used in these therapies may
also be in a variety of forms. These include, for
example, solid, semi-solid and liquid dosage forms,
such as tablets, pills, powders, liquid solutions or

10 suspensions, suppositories, injectable and infusible
solutions, and genetic therapy. The preferred form
depends on the intended mode of administration and
therapeutic application. Modes of administration may
include oral, parenteral, subcutaneous, intravenous,

15 intralesional or topical administration. The
compositions also will preferably include conventional
pharmaceutically acceptable carriers and may include
other medicinal agents, carriers, genetic carriers,
adjuvants, excipients, etc., e.g., human serum albumin

20 or plasma preparations. Preferably, the compositions
are in the form of a unit dose and will usually be
administered one or more times a day.

The following are examples which illustrate
the LT-ß and the LT-α/LT-ß complex of this invention

25 and the methods used to characterize them. These
examples should not be construed as limiting: the
examples are included for purposes of illustration and
the present invention is limited only by the claims.

EXAMPLES

30   We used the following experimental procedures
in the examples:

Antisera

Recombinant human LT-α (rLT-α) was expressed and secreted by a stably transfected chinese hamster ovary (CHO) cell line into serum-free conditioned

5   media. We purified the secreted rLT-α from the serum-free conditioned media by a series of Sepharose S, .lentil lectin-sepharose and FPLC Mono Q column chromatography steps. The properties of the CHO cell-derived rLT-α preparation have been described.

10   [J. Browning et al., J. Immunol., 143, 1859 (1989)]. We immunized two rabbits (4 and 5) by a lymph node procedure [M. Sigel et al., "Production Of Antibodies By Inoculation Into Lymph Nodes," Met. Enz., 93, 3 (1983)] with 25 μg of native rLT-α in complete Freund's

15   adjuvant. A third rabbit (6) was immunized via the same route with 25 μg of denatured rLT-α in complete ·Freund's adjuvant. We prepared denatured rLT-α by SDS-PAGE followed by electroelution into 0.1% SDS-carbonate buffer.

20   Using the above methods, three anti-rLT-α antisera were generated, two directed against native rLT-α and a third against SDS-denatured rLT-α. The antisera raised by immunization with native protein (rabbits 4 and 5) could neutralize a 50 unit/ml

25   solution at a dilution of 1:2000-5000. The serum raised against denatured rLT-α (rabbit 6) lacked neutralizing titer, but was weakly reactive with rLT-α on a Western blot. None of the antisera could neutralize r-human TNF nor could they recognize r-human

30   TNF bound to an ELISA plate except for a very weak titer in the antiserum from rabbit 6. Only antiserum from rabbit 6 was capable of recognizing rLT in a western analysis.

We immunized a fourth rabbit with recombinant

35   human TNF. We prepared the polyclonal anti-rTNF rabbit

serum via a classical immunization scheme using recombinant human TNF (E.coli derived [D. Weir et al., Handbook Of Experimental Immunology In Four Volumes, Chapter 8 "Immunization Of Experimental Animals"]) in

5 complete Freund's adjuvant followed by a boost in incomplete Freund's adjuvant. The serum raised against rTNF by immunization had a good neutralizing titer. A neutralizing monoclonal antibody to TNF has been described [Liang et al., "Production And

10 Characterization Of Monoclonal Antibodies Against Recombinant Human Tumor Necrosis Factor/Cachectin," Biochem. Biophys. Res. Comm., 137, 847 (1986)]. Pre-immunization serum was collected from all animals for use as controls.

15 Cell Growth and T cell Activation

All cells were obtained from the American Type Culture Collection (ATCC), except for the LT-α transfected Chinese hamster ovary (CHO) line that was described previously [Browning, J. Immunol., 143,

20 pp. 1859-1867 (1989)].

Cells were grown in RPMI 1640 supplemented with 1% glutamine, 10 mM HEPES buffer, pH 7.5, penicillin/streptomycin and 10% fetal bovine serum (Hyclone-defined) (designated "complete RPMI"), except

25 for the transfected CHO cells which were grown in Dulbecco's modified Eagle's medium supplemented as above. The human T cell hybridoma, II-23, was a result of a fusion of the human CEM tumor line with activated peripheral T lymphocytes and was further subcloned

30 (II-23.D7) [C. Ware et al., "Human T Cell Hybridomas Producing Cytotoxic Lymphokines: Induction of Lymphotoxin Release And Killer Cell Activity By Anti-CD3 Monoclonal Antibody Or Lectins And Phorbol Ester," Lymph. Res., 5, 313 (1986)]. Human peripheral blood

lymphocytes (PBL) were drawn into heparinized glass
tubes, isolated by Ficoll-Hypaque centrifugation,
washed and resuspended in complete RPMI medium. We
treated PBL at 2×10^6 cells/ml with a 1:500 dilution

5   of OKT3-conditioned medium (~2 ng/ml final) in the
presence of 1 μg/ml indomethacin and, in some
experiments, with 10 ng/ml rIL-2 (Biogen, Inc.,
Cambridge, MA)). Human CTL-clones were generated as
described [L. Green et al., "Cytotoxic Lymphokines

10   Produced By Cloned Human Cytotoxic T Lymphocytes," J.
Immunol., 135, 4034 (1985)] and activated either with
irradiated stimulator cells (antigen) or a combination
of anti-CD2 monoclonal antibodies (T11$_2$ + T11$_3$)
provided by E. Reinherz.

15   Flow Cytometry

We resuspended cells in RPMI 1640 medium with
10% fetal bovine serum (FBS), 0.1% sodium azide and 0.1
mg/ml human IgG at 0°C. Following preincubation with
the human IgG, we added additional media containing the

20   desired antisera. Typically the cells were incubated
with a final dilution of the anti-rLT-α and anti-rTNF
sera of 1:200 for 60-90 min. We washed cells twice
with Dulbecco's phosphate buffered saline (PBS) and
then incubated them with a 1:500 dilution of

25   fluorescein-labeled goat anti-rabbit IgG (Cappel
Durham, N.C.) in the above medium for a minimum of
60 min. Cells were then washed once and either
analyzed directly or, in some cases, analyzed following
fixation for 10 min. at 0°C with 0.5% paraformaldehyde.

30   We performed two color analyses as above, except that
we added phycoerythrin labeled leu-4, leu-2, leu-M3 or
leu-16 or leu-19 (Becton-Dickinson, Mountain View, CA)
at the second antibody stage. The comparison of
surface-bound LT-α with IL-2 receptor levels was done

with separate single color analyses with fluorescein-
labeled anti-IL-2 receptor (CD25) antibody (Becton-
Dickinson, Mountain View, Ca.). Analyses were
performed with a FACStar instrument (Becton-Dickinson).

5 Adsorption of Neutralizing Anti-rLT-α
Antibodies by Activated II-23.D7 Cells

We stimulated II-23.D7 and U937 premonocytic
cells at 1 x 10^6 cells/ml for 8 hours with 10 ng/ml of
PMA in complete RPMI medium. We washed the cells (1 x
10 10^8) three times in medium and aspirated the
supernatant to obtain a dry pellet. The cells were
then resuspended in 1 ml of medium containing a 1:1000
dilution of anti-rLT-α serum (from rabbit 4) and
incubated on ice for 1.5 hours with mixing. Cells were
15 cleared from the antiserum by centrifugation. We mixed
the absorbed antiserum (both pre- and post-immune) with
an equal volume (50 μl) of medium containing 15 U/ml of
rLT-α and incubated for 20 minutes at room temperature.
The mixtures were diluted serially into medium and
20 added to L929 cells (in 0.1 ml) and incubated a further
24 hours. We assessed cell viability by the MTT assay
as described [L. Green et al., "Rapid Colorimetric
Assay For Cell Viability: Application To The
Quantitation of Cytotoxic And Growth Inhibitory
25 Lymphokines" Jour. Immunol. Meth., 70, 257-268 (1984)].

^{35}S-Methionine or ^{35}S-Cysteine
Metabolic Labelling of T cells

We transferred cells into either cysteine-
free or methionine-free RPMI 1640 supplemented with
30 penicillin/streptomycin, glutamine, 10 mM HEPES pH 7.5,
10% v/v dialyzed FBS and 2% v/v conventional RPMI (cold
carrier addition). We adjusted the cell concentration
to 2-3 x 10^5 cells/ml and added ^{35}S-methionine or ^{35}S-
cysteine to the appropriate medium to a level of 100-

200 μCi/ml. In the case of freshly activated PBL, the
supernatants were gently removed, and the cells were
centrifuged, resuspended in labelling medium and added
back to the original adherent population. Following a
5 12-18 hour labelling period, we washed and lysed the
cells as described below. With the PBL, cells were
removed by pipetting and the adherent population
partially removed by treatment with 5 mM EDTA in PBS.

Immunoprecipitations

10   To 0.2-0.5 ml of labeled cell lysate we added
2-4 μl of rabbit serum. The sample was left for 1-2
hours at 4°C. We then added a 60 μl aliquot of a 60-
7.5% suspension of washed Protein A sepharose
(Pharmacia, Piscataway, N.J.) and rocked the sample for
15 6-18 hours at 4°C. We washed the Protein A sepharose
pellets 3 times with 1% NP-40 in calcium/magnesium free
PBS and resuspended them in 50 μl of Laemmli SDS
loading buffer. Typically a single lysate sample was
cycled through sequential immunoprecipitations with
20 preimmune anti-rLT-α serum, anti-rTNF antiserum and
finally post immune anti-rLT-α antiserum. In one set
of experiments, we added 5 mM $CaCl_2$ and $MnCl_2$ to the
lysate and the lysate was rocked overnight with 75 μl
of 75% suspension of washed lentil lectin-sepharose.
25 The sepharose was washed twice with NP-40/PBS and then
eluted with 3 consecutive additions of 75 μl of 1% NP-
40/PBS with 0.25 M α-methyl mannoside. We subjected
the pooled washes to the immunoprecipitation protocol.

Rabbit anti-rLT Affinity Column

30   We purified the immunoglobulin fraction from
the anti-rLT serum (from rabbit 4) using Protein A
sepharose with acid pH elution. The eluted IgG-
containing fractions were dialyzed against PBS and

concentrated by amicon filtration. The anti-rLT-α-IgG
solution (15 ml of 6 mg/ml) was coupled to 8 ml of
Affi-gel 10 resin (Biorad, Richmond, Ca.) as per
instructions. We prepared·an identical affinity column
5   using nonspecific rabbit IgG (Cappel, Durham, N.C.).
Both columns were washed with PBS, 1 M acetate pH 3.0
with 1% NP-40 and finally with lysis buffer lacking
protease inhibitors.

Initial Purification of LT-ß and LT-α

10   We grew II-23.D7 cells (15 1) to a density of
5 x 10^5 cells/ml and added phorbol myristic acetate
(PMA) to give a final concentration of 25 ng/ml. After
24 hours, we harvested the cells and washed them into
cold serum-free RPMI medium. To the chilled cell
15   pellet containing 7 x 10^9 cells we added 100 ml of ice-
cold lysis buffer (50 mM HEPES pH 7.5, 1% v/v NP-40, 2
mM EDTA, 0.15 M NaCl and 0.1% sodium azide) to which 5
mM benzamidine, 1 mM phenyl methyl sulfonyl chloride
(PMSF) and 0.25 mM N-ethyl maleimide (NEM), 10 μg/ml
20   soybean trypsin inhibitor, 0.7 μg/ml pepstatin and 10
μg/ml aprotinin had been freshly added. We gently
homogenized the cells in a dounce homogenizer and
centrifuged the lysate at 10,000 x g for 10 minutes.
We centrifuged the supernatant at 60,000 x g for 90
25   minutes and collected the supernatant.˙
             To the supernatant from the high speed
centrifugation we added 5mM $CaCl_2$ and 5mM $MnCl_2$. The
supernatant was then loaded onto a 20 ml lentil-lectin
sepharose column (Pharmacia, Piscataway, N.J.)
30   equilibrated in lysis buffer plus $CaCl_2$ and $MnCl_2$. We
washed the column with lysis buffer (with $CaCl_2$ and
$MnCl_2$) and then eluted the column with lysis buffer
containing 0.25 M α-methyl mannoside.

We pooled the lentil lectin eluate fractions
to give a volume of 50 ml and loaded them directly onto
a 2 ml rabbit nonspecific IgG sepharose affinity
column. We connected this column directly to a 2 ml
5   rabbit anti-rLT-α sepharose affinity column. We washed
both columns with the same lysis buffer with EDTA,
followed by lysis buffer wherein the 1% NP-40 had been
replaced with 1% w/v MEGA-8 (Octanoyl-N-methyl
glucamide, Boehringer-Mannheim, Indianapolis, IN.).
10   We eluted the washed columns individually
with 1% MEGA-8, 50 mM glycine pH 2.5, 0.05 M NaCl, 5 mM
benzamidine, and 2 mM EDTA. We pooled the first 20 ml
following the pH shift, lyophilized the pool and
resuspended it in 1 ml of water with 0.05% SDS, and
15   dialyzed it against 10 mM HEPES pH 7.5, 0.05% SDS and
0.1% MEGA-8. We dried the dialyzed fractions on a
speed-vac and resuspended them in 0.15 ml of water. We
mixed aliquots with Laemmli loading buffer and
electrophoresed them on SDS-PAGE. The LT-β and LT-α
20   proteins were visualized by silver staining.

Iodination of II-23.D7 Cell Surface

Either control or PMA-induced II-23.D7 cells
were washed extensively in calcium/magnesium-free PBS,
treated with 1 mM PMSF and 0.25 mM NEM and then washed
25   twice. To a 12 x 75 mm glass tube that was coated with
50 μg of iodogen (Pierce) we added 0.3 ml of cells (1 x
10^7 total) and 1-2 mCi of [125]sodium iodide. Cells were
left with periodic swirling for 25 minutes at room
temperature, washed 3 times in PBS with 10% FBS and
30   resuspended in lysis buffer as described above. We
then removed the nuclei with a 2 minute centrifugation
in an Eppendorf centrifuge. We then centrifuged the
supernatant an additional 15 minutes. The cleared

supernatant was subjected to the immunoprecipitation protocol.

1-Dimensional CNBr Peptide Mapping

5 We electrophoresed samples on a 12% acrylamide SDS-PAGE Laemmli system gel for a short distance and excised the appropriate gel sections. We soaked the gel slices for 1 hour in 1.0 ml of 0.1 N HCl, 0.2% 2-mercaptoethanol with 15 μl of 700 mg/ml fresh CNBr in 90% formic acid. The slices were then

10 removed and washed for 5 minutes with 0.1 M Tris-Cl pH 8.0, 5 min with 25 mM Tris-Cl pH 8.0 and finally 10 min with 1x Laemmli SDS-PAGE loading buffer. We loaded the slices onto a 15% SDS-PAGE Laemmli gel with a 12% acrylamide stacking gel. We visualized the peptide

15 bands by silver staining or autoradiography of the dried gel.

Reimmunoprecipitation

Reimmunoprecipitation of SDS-PAGE-separated antigens was carried out by excising labeled bands from

20 gels, rehydrating them for 10 minutes in TBS, 0.2% SDS, and then dicing the gel slices into small pieces. The proteins were eluted by incubation in 1 ml TBS, 0.2% SDS for 8 hours at room temperature, with rotation. After elution the gel pieces were removed by

25 centrifugation, and NP-40 was added to the supernatant to a final concentration of 2%. The eluted proteins were then immunoprecipitated as above, and reanalyzed by SDS-PAGE.

Isoelectric Focusing (IEF)

30 Two-dimensional IEF was performed essentially as described by P. H. O'Farrell [J. Biol. Chem., 250, 4007-4021 (1975)]. ^{125}I-labeled antigens were

immunoprecipitated from II-23.D7 cell extracts, and the immunoprecipitated proteins were eluted by heating at 100°C for 5 minutes in 100 μl O'Farrell sample buffer containing 9.5 M urea. The eluted proteins were then
5   focused (first dimension) on 14 cm x 3 mm tube gels possessing a 2% final concentration of ampholines (range pH 3-10, Sigma) at room temperature for 16 hours at a constant voltage (400 V). The second dimension was 12% SDS-PAGE.

10   For IEF under native (non-denaturing) conditions, ^{125}I-labeled cell extracts were focused directly on tube gels identical to those above except for the presence of urea. The labeled extract (200 μl volume) was centrifuged at 100,000 x g (30 psi,
15   airfuge) for 10 minutes prior to loading onto the tube gel. The focusing was performed at 4°C under the same conditions as described above. The tube gel was then removed and sliced into 1 cm sections, and the proteins were eluted by incubating each slice in 1 ml TBS, 2%
20   NP-40, 2 mM PMSF for 8 hours at room temperature, with rotation. The supernatants containing the eluted proteins were then immunoprecipitated and analyzed by SDS-PAGE. The pH gradients for both the denatured and native tube gels were determined by measuring the pH's
25   of individual slices from gels run in parallel.

T Cell Proliferation Assays

        We isolated and resuspended PBL in complete RPMI as described above except for the substitution of fetal bovine serum with 10% human autologous serum, 1
30   μg/ml indomethacin and 50 U/ml polymyxin B. In the MLR experiments, autologous serum was the responder's serum. We irradiated stimulator cells from a different donor with 3000 rads. We preheated rabbit sera for 1 hour at 56°C, and diluted and sterile filtered the sera

prior to use in proliferation assays. Cells (1 x 10^5 total) in 0.2 ml in a round bottom 96-well plate were treated with either 5 μg/ml phytohemagglutinin, 1-2 ng/ml OKT3 or 1.5-2 x 10^5 irradiated stimulator cells

5   in the presence or absence of various antisera or cytokines. After 3 days (PHA or OKT3 activation) or 5 days (MLR), cells were pulsed with ^{3}H-thymidine, harvested and counted.

Further Purification of LT-α and LT-β

10   We grew II-23.D7 cells in RPMI medium with 10% fetal bovine serum and we harvested the cells from . 50 1 RPMI medium and resuspended them in medium at a concentration of 4 x 10^6 cells/ml and we added 50 ng/ml phorbol myristoyl acetate (PMA). After activation for

15   6 hours we harvested the cells by centrifugation and washed them with Dulbecco's phosphate buffered saline. We suspended the final cell pellet of 4 x 10^{10} cells in 200 ml of cold lysis buffer (50 mM HEPES buffer, pH 7.0; 0.1 M NaCl, 10 mM EDTA, 5 mM benzamidine, 10 μg/ml

20   each of soybean trypsin inhibitor, aprotinin, chymostatin, leupeptin, antipain, 1 μg/ml pepstatin and 1 mM phenylmethyl sulfonyl fluoride) and passed the pellet once through a nitrogen cavitator. We centrifuged the lysed the cells at 40,000 rpm for 60

25   minutes in a 50.2 Ti rotor and discarded the supernatant. We extracted the pellet overnight in 120 ml of lysis buffer with 1% w/v Nonidet P40 detergent and then centrifuged it as above.

We added the supernatant containing the

30   detergent solubilized membrane proteins to 2 ml of affinity resin composed of monoclonal anti-lymphotoxin (anti-tumor necrosis factor-β from Boehringer Mannheim) coupled to Affi-gel 10 (BioRad) and rocked the suspension overnight. We collected the resin into a

small column and washed it with 50 mM HEPES, pH 7.0
with 1% Nonidet P40, and then with the same buffer with
1% w/v MEGA-8 (Boehringer Mannheim). We eluted the
bound proteins with 1% MEGA-8 in 50 mM glycine buffer

5 pH 2.5 and the fractions immediately neutralized with
Tris base. We determined the presence of p33 and LT in
the fractions by SDS-PAGE analysis and silver staining.
We pooled factions containing these proteins and added
SDS to a final concentration of 0.1% w/v and we

10 dialyzed the pool against 0.1x Laemmli sample buffer
(multiple changes to remove the MEGA-8 detergent). We
lyophilized the dialyzed solution to dryness and
resuspended it in 1/10th the original volume of water.
We ran the sample on an SDS-PAGE gel, blotted onto a

15 ProBlot membrane (Applied Biosystems) and stained with
coomassie blue dye.

This scheme allows one to purify LT-ß to a
band on a blot. It should be possible for anyone
skilled in the art to separate the proteins eluted from

20 the affinity resin by ion exchange chromatography. For
example, the complex can be dissociated with urea and
the LT-α and LT-ß proteins can be separated by, e.g.,
MONO Q FPLC (Pharmacia) anion exchange chromatography
in Tris-Cl buffer pH 8.0 with 1% nonionic detergent

25 (e.g., MEGA-8, Boehringer-Mannheim) and urea, using a
salt gradient elution. This chromatographic technique
separates on the basis of differing charges on the
proteins. The two proteins are separable in an
isoelectric focusing experiment (see, supra) on the

30 basis of charge differences, wherein urea is used to
dissociate the LT-α/LT-ß complex. Such a combination
of affinity chromatography, dissociation in
urea/nonionic detergent and ion exchange chromatography
allows purification of soluble LT-ß or the LT-α/LT-ß

35 complex.

Peptide Sequencing Assays

We excised the LT-ß and LT-α bands from the Problot and loaded them into a protein sequencer. We obtained N-terminal sequence information by Edman
5 degradation with a model 470A Applied Biosystems sequencer coupled to a 120A PTH amino acid analyzer. LT-ß was purified by immunoaffinity-chromatography as described above and tryptic fragment sequence was obtained. [See Abersold et al., "Internal Amino Acid
10 Sequence Analysis Of Proteins Separated By One Or Two-Dimensional Gel Electrophoresis After In Situ, Protease Digestion On Nitrocellulose," PNAS, 84, 6970-6974 (1987)]. That is, protein on the blot was digested with trypsin in situ followed by reverse phase HPLC
15 resolution of the digested peptides. The resulting N-terminal and internal tryptic fragment peptides were then sequenced by Edman degradation. The sequencing of the N-terminal and internal peptides designated as T105, T87/88, T100 and T67 are shown in Fig. 13.

20 ## Construction of Oligonucleotide Probes

From the sequence of T87/88 the following antisense probes were designed:

    1368 GTYTCNGGCTCYTCYTC [SEQ ID NO:9]
    1369 GTYTCNGGTTCYTCYTC [SEQ ID NO:10]
25 and synthesized by standard methods. [See, e.g., J. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2ed. (1989)].

Preparation Of An Induced II-23 cDNA Library

We prepared a cDNA sublibrary as follows:
30 We stimulated II-23.D7 cells for six hours with 50 ng/ml PMA to ensure the presence of LT-ß mRNA. We isolated the mRNA from these cells and reverse-

transcribed it into cDNA using techniques well known to the art. [B. Seed and A. Aruffo, "Molecular Cloning Of The CD2 Antigen, The T-Cell Erythrocyte Receptor, By A Rapid Immunoselection Procedure," PNAS, 84, 3365-3369

5   (1987)]. Using standard procedures, we ligated double stranded cDNA to a NotI-BstXI linker/adaptor having the following sequence:

          5' GCG GCC GCT TTA GAG CAC A 3' [SEQ ID
     NO:12]
10   3' CGC CGG CGA AAT CTC   5'

We then size-selected the cDNA on a 4.2 ml 5-20% potassium acetate gradient, 2 mM EDTA, 1 μg/ml ethidium bromide, in a Beckman® SW60 Rotor for 3 hours at 50,000 rpm at 22°C according to standard methods. We

15 pooled the cDNA fragments of greater than 500 base pairs. Then we prepared the vector, pCDM8 (a gift from Brian Seed (Massachusetts General Hospital). We digested this plasmid with BstXI. To remove the 400 base pair stuffer fragment we centrifuged the mixture

20 on a potassium acetate gradient, as above, and isolated the large fragment. We further purified this fragment by agarose gel electrophoresis, and then ligated the cDNA to the vector. In this way, we created recombinant DNA molecules containing DNA sequences for

25 mRNA expressed in induced II-23.D7 cells. We used these plasmids to transform E.coli MC1061 P3. The result was a collection of over 1×10^6 recombinant clones comprising a cDNA library for PMA induced II-23.D7 mRNA.

30 Screening and DNA Sequencing of Clones

          The pCDM8 II-23.D7 library was screened with ^{32}P labelled oligomer 1368 and positive clones were isolated following washing with 3M tetramethylammonium chloride at 50°C. [J. Sambrook et al., Molecular

35 Cloning, A Laboratory Manual, (1989); Jacobs et al.,

"The Thermal Stability Of Oligonucleotide Duplexes In
Sequence Independent In Tetraalkylammonium Salt
Solutions: Application To Identifying Recombinant DNA
Clones", Nucleic Acids Research, 16, 10, 4637-4649
5   (1988)].   Several clones containing 0.9 kb inserts were
subject to dideoxynucleotide DNA sequence analysis
[Id.].

Expression of LT-β cDNA

        The pCDM8/LT-β clone 12 or a control plasmid,
10 clone 4 (pCDM8 with an irrelevant cDNA insert), was
introduced by electroporation into CHO dhfr⁻ and a CHO
cell stably transfected with human LT-α. After three
days, cells were removed with Ca/Mg-free Hank's
solution with 5 mM EDTA and stained for FACS analysis
15 as described above using either 10 μg/ml control IgG_1
or anti-LT monoclonal antibody (Boehringer-Mannheim)
followed by labelling of bound immunoglobulin with
either a FITC or phycoerythrin labelled goat anti-mouse
preparation.   In other experiments, COS cells were
20 electroporated with either clone 4 or clone 12 LT-β
cDNA in pCDM8 in the presence or absence of an equal
amount of human LT-α cDNA also in the pCDM8 vector and
stained for FACS analysis after three days as above.

Northern Analysis of LT-β Expression

25   Poly A+ RNA was isolated from either II-23.D7
cells or peripheral blood mononuclear cells (PMBC)
using the Fast-Track™ system provided by Invitrogen.
Northern blots were prepared using 2 μg/lane of RNA and
electrophoresis on a formamide gel essentially as
30 described in J. Sambrook et al., Molecular Cloning, A
Laboratory Manual, (1989), followed by transfer onto
Gene Screen nylon membrane and UV crosslinking. Blots
were probed with random primed BstEII/Xmn-I fragment of

the LT-ß cDNA which had been gel purified, or a
fragment of human LT-α or actin. II-23.D7 cells were
induced with 50 ng/ml PMA for varying times and both
LT-α and LT-ß expression was found to be induced. PBMC
5 were either cultured in RPMI medium alone or in the
presence of 1000 units/ml of IL-2 or with OKT3 to
activate the T-cells.

Determination of the 5' End of LT-ß

The 5' mRNA sequence was determined by primer
10 extension analysis. [B. Wallner et al., Nature
320,77-81 (1986).] Primer extension using an
oligonucleotide primer (probe 360-121
5'GACAGTGATAGGCACCGCCAGCAACAA -3') [SEQ ID NO:13]
yielded a roughly 128-130 bp product that upon
15 sequencing using Maxam and Gilbert methodology
[A. Maxam and W. Gilbert, "Sequencing End-Labeled DNA
With Base-Specific Chemical Cleavages," Methods In
Enzymology, 65, 499 (1988)] showed the transcriptional
start site to be 7-9 bp upstream of the methionine ATG.
20 The expression exhibited by clone 12 in transient
experiments indicates that one or both of the Leu-4 or
Leu-6 start site is functional. To verify the 5' mRNA
sequence, a cosmid clone, 031A [Spies et al., Science
243, 214 (1989)], was digested with several restriction
25 enzymes, electrophoresed, blotted and probed with a BST
E2/Xmn-1 fragment of the LT-ß cDNA. The cosmid
contained the LT-ß gene within a 6 kb EcoR1 fragment
which was subcloned into a pUC derivative called
pNN 109 which contained a kanamycin resistance gene.
30 Dideoxynucleic acid sequencing gave the entire genomic
sequence.

Example 1

T cells Express LT-related
Epitopes on Their Surfaces

Under the conditions described above, we
5 activated human peripheral mononuclear cells (PMN) with
OKT3 monoclonal antibody and, after two days in
culture, we analyzed them for expression of LT-α/LT-β
complex related forms using flow cytofluorometric
analysis. In one experiment, the results of which are
10 shown in Figure 1, we cultured fresh PBL for 3 days
with OKT3 and IL-2 and stained them with a 1:200
dilution of antisera to native rLT-α ("LT-4" and "LT-5"
panels on Figure 1, from rabbits 4 and 5 respectively),
denatured rLT-α ("LT-6" panels on Figure 1, from rabbit
15 6) and native rTNF ("TNF" panel on Figure 1, from
rabbit 7). We stained cells with postimmune serum
(solid lines on Figure 1 panels) or with preimmune
serum from each animal (dotted lines on Figure 1
panels). Figure 1 shows that only anti-rLT-α sera from
20 rabbits 4 and 5 recognized epitopes on the activated
peripheral T cells.

In the experiment shown in Figure 2, we
treated II-23.D7 cells with or without 10 ng/ml PMA for
15 hours and stained them as described in the Figure 1
25 experiment, with rabbit 4 anti-rLT-α postimmune serum
(solid line on Figure 2 panels) or with rabbit 4
preimmune serum (dotted line on Figure 2 panels). As
shown in Figure 2, we found that the T cell hybridoma
II-23.D7, which synthesizes LT-α upon phorbol ester
30 (PMA) stimulation, expressed surface LT-related
epitopes upon PMA activation.

To establish that the LT-α-related epitopes
on T cells were related to LT and not to some
contaminant in the CHO-cell derived recombinant LT-α
35 preparation, we treated a 1:1000 dilution of antiserum

from rabbit 4 with PMA-activated, washed II-23.D7 or
U937 cells. In the experiment shown in Figure 3, we
treated a 1 ml sample of anti-rLT-α sera (1:1000 anti-
LT-4) with either no cells (- -), 1 x 10^8 U937 cells

5   (-O-), 1 x 10^8 (- -) PMA-activated II-23.D7 cells or 1
x 10^7 (- -) PMA-activated II-23.D7 cells. We added
dilutions of absorbed antisera to a limiting amount of
rLT-α in a L929 cytotoxicity assay such that a 1:4000
final dilution was present in the first well. This

10 assay measures the ability of LT-α to kill a mouse
fibroblast cell line, L929, within a 24 hour period
[L. Green, J.L. Reade, C.F. Ware, "Rapid Colorimetric
Assay for Cell Viability: Application to the
Quantitation of Cytotoxic and Growth Inhibitory

15 Lymphokines," J. Immunol. Methods, 70, 257 (1984)].
After 24 hours, we assessed cell viability using a MTT
readout. Plotted on Figure 3 is optical density (which
is proportional to cell viability) vs. the dilution of
absorbed antisera. Data represent the average of

20 duplicate wells and duplicates generally were within
the range defined by the symbol. As shown in Figure 3,
analysis of the neutralizing titer of the absorbed
antisera in the standard L929 cytotoxicity assay
demonstrated that the activated II-23.D7 cells removed

25 the LT-α neutralizing antibodies, whereas U937 cells
were ineffective. These data indicate that the
antigenic structures on the membrane surface are
actually related to LT-α.

We subjected the hybridoma II-23.D7 to a
30 number of further treatments to examine a number of
trivial explanations for the apparent existence of LT-α
related epitopes on T cell surfaces. First we ruled
out the possibility that LT-α:antibody complexes in the
antisera could bind to TNF/LT-α receptors on the

35 hybridoma. Both TNF and LT-α have trimeric structures

which could allow for the presence of antibody binding epitopes within the complex. However, prior saturation of the cellular TNF receptors with soluble TNF or LT-α had no effect on the surface staining. Such saturation
5 should have prevented such an immune complex from binding to such a receptor.

A pH 3 lactic acid treatment, which can release bound TNF from its receptor, had no effect on the signal, suggesting that the LT-α is not receptor
10 bound. However, experiments utilizing ^{125}I-LT-α binding to II-23.D7 cells indicated that receptor bound LT-α was more difficult to remove from its receptor at acidic pH's than TNF.

Mild trypsinization of the cells prior to
15 staining led to a loss of the signal, indicating that the epitope is a protein. To determine whether surface-associated LT-α was phosphatidylinositol linked, the cells were treated with a phosphatidylinositol specific phospholipase C. Under
20 conditions where a PI-linked antigen, LFA-3, could be released [A. Peterson et al., "Monoclonal Antibody And Ligand Binding Sites Of The T Cell Erythrocyte Receptor (CD2)," Nature, 329, 842 (1987)], no effect was observed on the LT-α epitope.

25 We could not stain CHO cells stably transfected with the LT-α gene, either with or without prior PMA activation, indicating that antibodies to CHO derived contaminants in the original rLT-α used to immunize the rabbits were not present in sufficient
30 amounts to contribute to the staining of II-23.D7 cells. Likewise, antibodies generated against any fetal bovine serum proteins contaminating the LT-α preparation would be ineffective in staining T cells since the staining was performed in 10% fetal calf
35 serum.

Pretreatment of the anti-rLT-α serum with
rLT-α blocked the staining of LT-α forms on II-23.D7
cells whereas pretreatment with rTNF did not.

Example 2

5   Immunoprecipitation of LT-α-related
        Proteins on the T cell Hybridoma II-23.D7

         We surface iodinated PMA activated II-23.D7
cells and lysed and solubilized the cells in detergent.
Immunoprecipitation and SDS-PAGE analysis of the
10   labeled membrane proteins showed that two proteins were
recognized by anti-rLT-α antisera. Figure 4A shows the
results of SDS-PAGE analysis of the iodinated surface
proteins precipitated with either pre-immune (PRE) or
post-immune (POST) anti-rLT-α serum (from rabbit 4).
15   As shown in Figure 4A, we observed a 25-26 kD
molecular weight form ("LT-α") that correlated with the
expected size of LT-α, and we also saw an additional
form of approximately 33 kD ("LT-ß or p33"). Neither
the preimmune serum from the same rabbit (Figure 4A
20   column PRE) nor anti-rTNF rabbit serum were able to
immunoprecipitate any bands from the iodinated, PMA-
activated II-23.D7 cells.
         1-D partial CNBr peptide mapping of the
iodinated bands showed that the 25-26 kD form was
25   cleaved in a pattern identical to that of iodinated
rLT-α, thus identifying this band as LT-α. In the
experiment shown in Figure 4B, the 25-26 kD and 33 kD
bands from panel A were excised, subjected to limited
CNBr cleavage and electrophoresed on a SDS-PAGE system.
30   For comparison, cleavages of both rTNF and rLT-α
performed in parallel are shown in Figure 4B. The gels
were visualized by autoradiography. Lane 1 represents
rTNF, lane 2 represents rLT, lane 3 represents LT, and
lane 4 represents LT-ß. The increased sizes of the

CNBr fragments reflect the increased amount of carbohydrate on natural LT-β. The iodinated 33 kD form was not cleaved by CNBr (lane 4), indicating that it is different from the known LT-α gene product. rTNF was

5 not cleaved with CNBr (lane 1) due to the absence of methionine in this protein.

We undertook metabolic labelling with ^{35}S-methionine and ^{35}S-cysteine coupled with immunoprecipitation to characterize further these

10 LT-α-related surface forms. In the case of the TNF/LT-α pair, the distribution of cysteine and methionine allows one to distinguish both between TNF and LT-α and between forms with and without their signal sequences, as was exploited in studies on the

15 membrane TNF form [M. Kriegler et al., Cell, 53, pp. 45-53 (1988)]. In the case of the fully processed cytokines, i.e., secreted forms, TNF contains cysteine and not methionine, while LT-α contains only methionine and not cysteine. LT-α, however, has two cysteine

20 residues in the signal sequence domain and TNF contains a methionine residue in this N-terminal region. Separate cultures of II-23.D7 hybridoma cells were labeled with either ^{35}S-methionine or ^{35}S-cysteine and immunoreactive proteins were precipitated. In an

25 experiment, the results of which are shown in Figure 5, II-23.D7 cells were activated with 10 ng/ml PMA and simultaneously labeled for 8 hours with either ^{35}S-methionine or ^{35}S-cysteine. Both the medium and lysed cells were subjected to consecutive

30 immunoprecipitations with preimmune (rabbit 4) (P), anti-rTNF (T) and anti-rLT-α (rabbit 4) (L) sera in that order. Figure 5 shows an SDS-PAGE auto-radiographic analysis of the immunoprecipitates from either supernatants containing secreted proteins or the

35 washed cells. "s-TNF" marks the ^{35}S-methionine labeled

anti-rTNF immunoprecipitated band from the cells that was putatively assigned as the unprocessed 26 kD form of TNF. "Met" and "Cys" refer to the ^{35}S-labeled amino acid employed. Those lanes containing ^{35}S-cysteine

5 were exposed for longer periods of time than the lanes containing ^{35}S-methionine. In the supernatants from these cells (lanes labeled "secreted"), a 25 kD form of LT ("LT-α") was released following PMA treatments by those cells that were labeled with ^{35}S-methionine, but

10 not by those labeled with ^{35}S-cysteine. This pattern is expected for fully processed, secreted LT-α. Longer exposures showed trace amounts of TNF in the supernatant, and the incorporation of label was as expected for fully processed, secreted TNF. We

15 observed the expression of predominantly LT-α with low levels of TNF also at the mRNA level (Shamansky and Ware, unpublished observation). Analysis of the washed cells (lanes labeled "cellular") showed that both the 25-26 kD LT-α, along with the 33 kD LT-ß, were present.

20 The relative amounts of the 25-26 kD and 33 kD forms paralleled those observed using surface iodination. The 25-26 kD surface LT-α form lacked cysteine, indicating processing of the leader sequence. The 33 kD form incorporated both ^{35}S-methionine and

25 ^{35}S-cysteine. Longer exposures (not shown) of the film shown in Figure 5 revealed the presence of an anti-TNF immunoprecipitated band from the cells at about 26-27 kD. The band showed incorporation of both labeled cysteine and labeled methionine. The labelling was

30 stronger with cysteine. Since the cys:met ratio is 4:1 in the 26 kD-TNF form, this labelling pattern confirms the identity of this band.

The presence of LT-ß with LT-α in immunoprecipitates from cell lysates suggested that

35 either LT-ß is antigenically related to LT-α or that

LT-ß is bound to LT-α or both. To address this issue
25 kD and 33 kD bands from ^{35}S-methionine labeled cells
were immunoprecipitated with rabbit polyclonal anti-
rLT-α serum, eluted from excised gel slices and
5 subjected to reimmunoprecipitation with either anti-
rLT-α polyclonal serum or mAb. LT-α, but not LT-ß,
could be immunoprecipitated with either anti-rLT-α
antibodies suggesting that LT-ß is not antigenically
related to LT-α. These observations indicated that
10 LT-ß is physically associated with LT-α. We believe
that the 33 kD protein is unrelated antigenically to
LT-α and simply co-precipitated with LT-α.

With either surface iodination or metabolic
labelling, we were unable to detect either of the known
15 55 or 80 kD TNF/LT-α receptor forms associated with
LT-α or TNF. Presumably, this is because the receptors
are rapidly lost during activation of T cells.
[C. Ware et al., "Regulation Of The CTL Lytic Pathway
By Tumor Necrosis Factor," Cellular Immunity And The
20 Immunotherapy Of Cancer, UCLA Symposia on Molecular and
Cell Biology M.T. Lotze and O.J. Finn, Eds. Vol. 135,
pp. 121-128 (Wiley-Liss, Inc. New York) 1990].

Example 3

Biochemical Characterization of
25 ### Surface LT-forms on II-23.D7 Cells

We purified the LT-related forms on the
surface of PMA-treated II-23.D7 cells using affinity
chromatography. Using immunoprecipitation techniques,
we had noted that both of LT-ß and LT-α bound to lentil
30 lectin sepharose, indicating a glycoprotein structure.
We bound detergent solubilized PMA-treated II-23.D7
proteins to lentil lectin sepharose and eluted with
α-methyl mannoside prior to affinity purification. We
prepared both control IgG and anti-IgG columns to more

accurately assess those proteins specifically
recognized by the anti-rLT-α serum. Low pH elution of
the columns led to the release of about 100-200 ng of
the two LT forms from the anti-rLT-α column.

5   Figure 6A reflects SDS PAGE analysis of the
proteins eluted from anti-rLT-α affinity column
prepared from either pre-immune (PRE) or post-immune
(POST) rabbit sera. In Figure 6B, the 33 kD and 20 kD
bands from the gel in panel A were excised and

10   subjected to limiting CNBr cleavage and electrophoresed
on a SDS-PAGE system. For comparison, Figure 6B showed
CNBr cleavages of rTNF and rLT-α (CHO-derived)
performed in parallel. The gels were visualized by
silver staining. SDS-PAGE gels of the eluate resembled

15   closely gels of immunoprecipitated, surface iodinated
PMA-treated II-23.D7 cells, indicating that similar
proteins had been purified.

              During the affinity purification, the 25 kD
LT-α form appeared to be cleaved to a 19-20 kD form,

20   i.e., it now co-migrated with the intact recombinant
CHO cell-derived LT-α. The original isolation of
natural LT-α from the RPMI 1788 tumor line also yielded
an N-terminally cleaved "des-20" LT-α form. One-
dimensional CNBr digests of the affinity purified

25   proteins showed the cleaved 20 kD LT-α form to have a
CNBr cleavage pattern that presumably reflects the
truncated nature of this LT-α form. One of the
methionines is lost in the "des-20" LT-α form and hence
the cleavage pattern would be different from that of

30   the intact LT-α form. The 33 kD protein (LT-β)
generated a doublet upon CNBr cleavage, and from this
it was estimated that the single methionine must lie
within 5-20 residues from either the C- or N-terminus.
This cleavage pattern shows that the 33 kD protein is

35   significantly different from known LT forms. CNBr

cleavage analysis of the surface iodinated 33 kD
protein probably gave a similar result; however, the
resolution achievable with iodination was insufficient
to visualize the doublet. Staphylococcus V8 digestion

5   of the iodinated rLT-α, rTNF and II-23.D7 LT forms
showed the rLT-α and 25-26 kD II-23.D7 LT-α form to be
resistant to digestion, confirming the assignment of
this protein as LT-α. The 33 kD protein was cleaved
into several smaller fragments with the pattern

10   resembling closely that of rTNF.

In Figure 7, immunoprecipitated, surface
iodinated proteins were resolved on SDS-PAGE analysis
and the surface-associated 25-26 kD protein ("sLT-α")
and the 33 kD protein ("LT-ß") bands were excised.

15   Slices were digested with N-glycanase (N-Gly), with a
mixture of neuraminidase and O-glycanase (O-Gly), or
with all three enzymes. The digested slices were rerun
on SDS-PAGE and an autoradiogram of the dried gel is
shown. As shown in Figure 7, immunoprecipitation of

20   iodinated surface LT forms followed by digestion with
either or both N- and O-glycanases showed the 25-26 kD
LT-α form to contain an N-linked oligosaccharide. The
25-26 kD LT-α form contains only one N-linked site
which would correlate well with the size change upon N-

25   glycanase digestion. Likewise, the 33 kD form (LT-ß)
lost about 3 kD of size upon treatment with N-
glycanase, suggesting the presence of one N-linked
oligosaccharide. In contrast to the 25-26 kD LT-α
form, O-glycanase treatment did not affect the

30   molecular weight of LT-ß. The lack of cleavage by a
glycanase, however, is not definitive evidence for the
lack of a carbohydrate.

Example 4

Reimmunoprecipitation of LT-α

The coprecipitation of LT-ß with LT-α
suggested that these proteins are either antigenically
5   related or physically associated.   To address this
issue we tested whether or not SDS-PAGE separated LT-α
(p25) and LT-ß proteins could be immunoprecipitated.
LT-α and LT-ß labeled with ^{125}I or ^{35}S-Met were first
partially purified by immunoprecipitation and separated
10   by SDS-PAGE.   The labeled bands were excised,
rehydrated in buffer, and the proteins eluted.   The
eluted proteins were then subjected to a second round
of immunoprecipitation using either polyclonal or
monoclonal anti-rLT-α antibodies (Fig. 10).   Rabbit
15   anti-rLT-α reimmunoprecipitated LT-α ("p25", lane 2)
but not LT-ß (lane 3).   The anti-rLT-α mAb precipitated
LT-α (lane 5) and a 21 kD protein ("p21", lane 4),
which, as shown below, is a precursor of LT-α; however,
it did not precipitate LT-ß (lane 6).   The results
20   indicate that after LT-α and LT-ß are separated by SDS-
PAGE, both polyclonal and monoclonal anti-rLT-α
antibodies are capable of reacting with LT-α but not
with LT-ß.   This data provides evidence that LT-ß is
not antigenically related to LT-α.   However, we cannot
25   rule out the possibility that putative LT-ß cross-
reactive epitopes are lost after denaturation, whereas
the LT-α epitopes remain intact.

Figure 8 shows the results of reimmuno-
precipitation of ^{125}I-labeled and ^{35}S-Met-labeled p25
30   and p33 proteins eluted from SDS-PAGE gels.   LT-α and
LT-ß species from ^{125}I-labeled II-23.D7 cells (lanes
2,3) and ^{35}S-labeled cells (lanes 4,5,6) were eluted
from gel slices as described above.   The elutes were
immunoprecipitated with either the anti-rLT-α serum
35   (lanes 2,3) or the anti-rLT-α mAb (lanes 4,5,6), and

the reprecipitated proteins analyzed by SDS-PAGE and autoradiography. Lane 1 is a control lane for the identification of LT-α and LT-β.

Example 5

5   Isoelectric Focusing of LT-α and LT-β

Figures 9 and 10 (each including an autoradiograph (9A, 10A) and a calibration curve graphing migration distance vs. pH (9B, 10B)) depict isoelectric focusing analysis under denaturing (Fig. 9)
10   and native (Fig. 10) conditions. Two-dimensional gel analysis was carried out as described above on ^{125}I- labeled LT-α and LT-β that had been immuno- precipitated from II-23.D7 cell extracts. The 2-D gel analysis was performed under denaturing conditions in
15   the presence of urea (Fig. 9A). In contrast, native IEF was performed in 1% NP-40 without urea. ^{125}I- labeled II-23.D7 cell extract was first focused on a tube gel at 4°. After focusing, the tube gel was cut into 1 cm sections, the focused proteins eluted from
20   those sections, immunoprecipitated, and analyzed by SDS-PAGE (Fig. 10A). Immunoprecipitated material from gel lanes 1-12 correspond to tube gel slices 2-13. pH gradients were generated for both the denatured and native tube gels based on 1 cm gel increments. These
25   are also shown below each autoradiogram as 9B and 10B, respectively. Biochemically, LT-β and LT-α comigrate on a non-denaturing isoelectric focusing gel, but when the complex is dissociated with urea, the two proteins run separately. [See Figs. 9A, 10A] These
30   observations led us to conclude that LT-α and LT-β exist as a complex on the cell surface.

Example 6

Regulation of LT-α Expression

Table I set forth below, summarizes the
results of a survey using flow cytofluorometric
5   analysis of various cell types for the expression of a
surface form of LT-α.

Table I

Expression of LT-α and TNF Related Epitopes on the Surfaces of Different Cells

		Surface Expression of:	
Cell	Treatment	LT-α	TNF
Peripheral Mononuclear cells	Resting	+	−
	OKT3	++	−
Leu-4[+] (CD3)	Resting	+	−
	OKT3	++	−
	PMA	−	nd.
	IL-2	++	nd.
Leu-2[+] (CD8)	OKT3	++	nd.
Leu-3[+] (CD4)	OKT3	++	nd.
Leu-M3	Resting	−	+
	OKT3/LPS/IFN-γ	−	++
Leu-19[+] (NK)	IL-2 (LAKs)	++	nd.
Leu-16[+] (B's)	Resting	+/−	−
	PWM	+	nd.
CTL-clones	Control	+/−	−
	PMA	nd.	nd.
	Allogeneic Stim.	++	nd.
	Anti-T11 2+3	++	−
T cell Hybridoma (II-23.D7)	Control	−	−
	PMA	++	−
	PMA + A23187	++	−
Hut-78	Control	+	−
	PMA	+	−
C8166	Control	−	nd.
	PMA	+	nd.
RPMI-1788	Control	−	−
	PMA	−	−
rLT-producing CHO cell line	Control	−	−
	PMA	−	−
Jurkat		+/−	nd.
HL-60		−	−
U937		−	nd.
Raji		+	nd.
K562		+/−	nd.

The most striking observation from these studies was the restriction of surface LT-α expression to T and B cells. Leu-M3, a monocyte marker, and leu-4 (CD3) antibodies were used in two-color flow
5 cytofluorometric analysis to observe each cell population separately. There was an excellent distinction between surface TNF and surface LT-α in this analysis in that monocytes expressed only surface TNF whereas T cells displayed only surface LT-α. This
10 result is shown in Figure 11.

In the experiment depicted in Figure 11, PBL were treated for 8 hours with a mixture of LPS (1 μg/ml), Interferon-γ (200 U/ml) and OKT3 (1ng/ml) and then stained for LT-α (anti-rLT-α serum from rabbit
15 5) or TNF (anti-rTNF serum from rabbit 7) followed by FITC anti-rabbit labelling. Cells were counterstained with either phycoerythrin-leu-4, a pan T cell marker, or phycoerythrin leu-M3, a monocyte marker. "T cell panels" were gated for leu-4^{+} cells while the
20 "monocyte" panels were gated for leu-M3^{+} cells. Cells were stained with preimmune (dotted lines) or postimmune sera (solid lines). The monocytic tumor lines HL-60 and U937 did not stain for LT-α. By using two color flow cytofluorometric analysis, the T4 and T8
25 subclasses of activated PBL were found to display similar levels of surface-associated LT-α. In general, it appears that primary T-cells capable of expressing LT-α are also capable of displaying surface LT-α form(s).

30 Examination of three different human donors showed that a surface LT-α form was present on freshly isolated, resting peripheral T cells. In the case of PBL, OKT3 activation or simply IL-2 treatment of the cells led to increased expression. By using
35 fluorescence channel numbers, we have attempted to

quantitate both surface LT-α and IL-2 receptor (CD25)
expression during OKT3 activation. Maximal surface
LT-α induction by OKT3 appeared to precede the peak
expression of IL-2 receptor (TAC expression) in the
5   bulk culture, thus the surface-bound LT form (LT-α/LT-β
complex) appears to be an early T-cell activation
antigen. It was found that both anti-T11 and
allogeneic antigen were capable of causing the
appearance of LT-α on the surface of cloned cytotoxic T
10 cells. Likewise, PMA stimulation was necessary to
induce the appearance of LT-α on the surface of the II-
23.D7 hybridoma. It appears that T cell activation
increases surface LT-α form(s). Peripheral
lymphocytes, in contrast to the II-23.D7 hybridoma,
15 down-regulate surface LT-α form(s) very quickly
following PMA treatment. Likewise, in a two-color
analysis of OKT3 activated PBL populations, Dr^{+} cells,
which should include T cells in advanced stages of
activation, lacked surface LT-α form(s).
20   Activation of fresh PBL with high levels of
IL-2 generated lymphokine-activated killer cells (LAK
cells). As shown in Figure 12, two color flow
cytofluorometric analysis using anti-rLT-α and leu-19,
a NK/LAK cell marker, showed LAK cell expression of
25 surface LT-α forms to resemble the T cell hybridoma,
II-23.D7. In the experiment depicted in Figure 12, PBL
were cultured for 5 days with 20 ng/ml IL-2 and then
stained for a two color analysis with phycoerythrin
labeled leu-19 and anti-rLT (rabbit 5) as described
30 above. Figure 12 shows surface LT-α levels on leu-19[+]
cells that were stained with preimmune (dotted lines)
or postimmune sera (solid lines). Thus, LAK cells
appeared to have the highest levels of surface LT-α
forms of any primary cell type.

Example 7

Functional Relevance of Total TNF or LT-α to T cell Activation

To examine the functional relevance of TNF
5   and LT-α to the T cell activation process, we included
the rabbit anti-rLT-α and anti-rTNF sera in mixed
.lymphocyte response (MLR) and OKT3 activation assays.
MLR is a standard immunological assay which tests the
ability of an individual's T cells to recognize another
10 person's T cells as foreign and respond to their
presence by proliferating. Table II, set forth below,
presents data from MLR experiments using various
responder/stimulator combinations.

TABLE II

Effects of LT and TNF Antibodies on a 5-Day Mixed Lymphocyte Culture

Cells[b]	Addition	^{3}H-Thy. (S.D.) % Change[a] cpm x 1000	
Responder A	none	4.7 (0.8)	-
Stimulator B	none	4.8 (0.5)	-
A + B	none	20.3 (3.6)	0%
A + B	r-TNF[c]	28.0 (1.4)	+49%
A + B	r-LT	32.8 (3.2)	+80%
Responder C	none	6.3 (0.8)	-
Stimulator B	none	4.8 (0.7)	-
C + B	none	30.0 (5.4)	0%
C + B	r-TNF	36.9 (6.0)	+28%
C + B	r-LT	36.1 (5.8)	+24%
Responder A	none	5.3 (0.6)	-
Stimulator D	none	1.5 (0.5)	-
A + D	none	22.2 (2.6)	0%
Responder D	none	7.8 (1.0)	-
Stimulator A	none	1.6 (0.3)	-
D + A	none	24.3 (7.0)	0%

Cells[b]	Addition	Preimmune	Postimmune
A + B	Anti-LT-4[d]	26.8 (2.4) +41%	8.6 (0.2) -74%
A + B	Anti-LT-5	27.4 (4.4) +45%	11.0 (1.2) -59%
A + B	Anti-LT-6	23.1 (1.0) +18%	25.7 (5.7) +35%
A + B	Anti-TNF	26.0 (2.2) +36%	12.6 (2.7) -49%
A + B	Anti-TNF mAb	15.1 (2.2) -33%	4.7 (0.7) -99%
C + B	Anti-LT-4[d]	41.1 (3.5) +44%	20.9 (5.8) -36%
C + B	Anti-LT-5	35.5 (6.9) +22%	17.8 (2.6) -49%
C + B	Anti-LT-6	39.4 (7.9) +38%	39.1 (5.3) +36%
C + B	Anti-TNF	39.8 (4.3) +39%	24.4 (3.2) -22%
C + B	Anti-TNF mAb[e]	37.8 (7.3) +31%	20.6 (1.8) -37%
A + D	Anti-LT-5	28.6 (2.1) +37%	12.5 (2.4) -59%
A + D	Anti-LT-5	32.8 (6.4) +63%	14.5 (3.6) -47%
D + A	Anti-LT-5	28.0 (1.2) +23%	20.8 (1.4) -21%
D + A	Anti-LT-5[f]	28.1 (2.1) +24%	19.2 (0.7) -31%

See notes below.

[a]Percent change refers to the increase or decrease in ^{3}H-thymidine incorporation after correction for a background of responder cells alone.

^{b}stimulator cells were irradiated with 3000 rads and are denoted with a "*". Low level proliferation was still evident in stimulator population. The ratio of responder to stimulator was 1/1.5.

5   ^{c}rTNF and rLT were added to a level of 10 ng/ml.

^{d}Antisera were heat inactivated for 1 hour at 56°C, filtered and used at a final dilution of 1:250.

^{e}The monoclonal anti-TNF was a purified mouse IgG$_{2a}$ antibody used at 2 μg/ml and the control in this case
10 was pure mouse UPC 10 (IgG$_{2a}$).

^{f}In these cases, the immunoglobulin fraction was purified from the serum and used at a final concentration of 50 μg/ml.

        As shown in Table II, the neutralizing anti-
15 rLT-α sera (rabbits 4 and 5) inhibited the
    proliferative response as assessed at five days whereas
    preimmune sera or the non-neutralizing anti-rLT-α
    (rabbit 6) sera had mild stimulatory effects. As
    previously reported [M. Shalaby et al., J. Immunol.,
20 141, 499 (1988)], polyclonal and a monoclonal anti-TNF
    preparations were also inhibitory. These assays were
    carried out under excess stimulator cell conditions and
    hence the inhibition may not be optimized. The serum
    levels employed in Table II are rather high, but in
25 other experiments (data not shown), antibody dilutions
    up to 1:1000 were still inhibitory. PHA or OKT3
    stimulated T cell proliferation was also inhibited to a
    lesser extent (data not shown). These data indicated
    that LT or LT-related epitopes on T cell surfaces may
30 be involved in T cell activation.
        A previous study using the MLR assay and
    neutralizing monoclonal antibodies implicated TNF but
    not LT-α in T cell activation and subsequent
    proliferation in this system. [M. Shalaby et al., J.
35 Immunol., 141, 499 (1988)]. In that study, monoclonal

anti-rLT-α antibodies had no effect on the MLR assay. Our studies indicate that neutralizing polyclonal anti-CHO-cell-derived-rLT-α sera were able to partially inhibit the MLR, suggesting a role for some form of LT

5   in this system. The reasons for this discrepancy are not clear, although there may be some differences in the nature of these antibody preparations. The monoclonal antibodies were generated against glutaraldehyde-cross linked natural LT-α (RPMI 1788

10 secreted), whereas polyclonal anti-rLT-α sera were prepared using native r-LT-α (recombinant CHO-cell derived) with Freund's adjuvant injected directly into . the lymph nodes. The depot effect in the success of the latter system was probably important considering

15 the difficulties reported in immunizing mice [T. Bringman et al., "Monoclonal Antibodies to Human Tumor Necrosis Factors Alpha and Beta: Application For Affinity Purification, Immunoassays, And As Structural Probes," Hybridoma, 6, 489 (1987)]. These data suggest

20 that the blocking effect of our polyclonal anti-rLT-α sera on the MLR is a result of recognition of the surface LT form(s) by the sera rather than recognition of the conventional soluble LT-α form.

Example 8

25 ## Purification And Initial Sequencing Of LT-α And LT-β

We obtained N-terminal sequence information for LT-α and LT-β by Edman degradation as described above. We found the sequence of the membrane associated LT-α band to be as follows: Leu Pro Gly Val

30 Gly Leu Thr Pro Ser. This sequence matches with the known sequence of secreted LT-α. The Edman degradation analysis revealed that the N-terminal portion of the associated LT-β protein included two possible amino acid sequences: Gly Leu Glu Gly Arg Gly Gln Arg Leu

Gln or Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln.
Subsequent DNA analysis confirmed the former sequence
except that glycine was incorrectly identified as
glutamine at cycle 7.

5

Example 9

Sequencing And Cloning LT-ß

As described above, we obtained sequences for
several peptides using the method of Abersold et al.
Two antisense 17-mer oligonucleotide probes
10 GTYTCNGGCTCYTCYTC [SEQ ID NO:9] and GTYTCNGGTTCYTCYTC
[SEQ ID NO:10] were synthesized to match a portion of
the sequence of one of those peptides, T-87/T-88.
Those probes were radiolabelled with ^{32}P. Northern
analysis (as described in J. Sambrook et al., Molecular
15 Cloning a Laboratory Manual, 2d ed. (1989)) showed that
probe 1368 (GTYTCNGGTCYTCYTC) [SEQ ID NO:10] hybridized
strongly to a 0.9-1.1 kb mRNA band that was strongly
induced in II-23.D7 cells which had been pretreated
with phorbol ester as previously described. A cDNA
20 library in the vector pCDM8 was constructed from poly
A+ mRNA isolated from II-23.D7 cells induced with PMA
for 6 hours. [See, B. Seed and A. Aruffo, PNAS, 84,
3365-3369 (1987)]. The library was screened with
labelled oligomer 1368 and positive clones were
25 isolated following washing with 3 M tetramethylammonium
chloride at 50°C. [See Jacobs et al., Nucleic Acids
Research, 16, 10, 4637-4649 (1988)]. Several clones
containing 0.9 kb inserts were subjected to DNA
sequence analysis. Clone pCDM8/LT-ß-12 (clone 12) was
30 found to contain the coding sequence. The other clones
were identical except for various 1-30 bp truncations
at the 5' end. One potential clone (clone 4) contained
a frameshift and was used as a control in transfection
experiments. A termination sequence, AATAAA at

position 862 was found just prior to a poly A
tract indicating that the entire 3' end had been
identified. The identified protein sequence encodes
for at least 240 amino acids with a calculated
5   molecular weight of 25,390 and a domain structure
typical of a type II membrane protein. The present
data suggest that the initiating CTG leads to a
processed N-terminus starting with gly 5 (met=1), i.e.,
the CTG encoded leucine is either not translated or the
10   leucine-4 residue is further processed yielding the
mature N-terminus obtained by amino acid sequencing.

The 33 kDa size of LT-ß results from N-linked
glycosylation as previously defined and this result is
corroborated by the presence of one potential N-linked
15   carbohydrate site in the amino acid sequence at a
position identical with a similar site found in the
CD40 ligand. A typical N-linked sugar residue can add
approximately 3-4 kDa MW, hence, the final molecular
weight is close to the observed 30-33 kDa.
20   No identical sequences were found within the
databases. There is one cysteine residue in the
extracellular domain and two methionines within the
last C-terminal 17 amino acids, in agreement with the
very limited cyanogen bromide cleavage pattern
25   exhibited by this protein.

Example 10

Expression Of LT-ß

The pCDM8/LT-ß clone 12 or a control plasmid,
clone 4 (pCDM8 with a non-functional cDNA insert), were
30   introduced by electroporation into CHO dhfr- and a CHO
cell stably transfected with human LT-α. After three
days, cells were removed with Ca/Mg-free Hank's
solution with 5 mM EDTA and stained for FACS analysis
as described above using either 10 μg/ml control IgG1

or anti-LT monoclonal antibody (Boehringer-Mannheim) followed by labelling of bound immunoglobulin with either a FITC or phycoerythrin labelled goat anti-mouse preparation.

5   In a different experiment, COS cells were electroporated with either clone 4 or clone 12 LT-ß cDNA in pCDM8 in the presence or absence of an equal amount of human LT-α cDNA also in the pCDM8 vector and stained for FACS analysis after three days as above.

10   Only COS cells expressing LT-α displayed surface lymphotoxin upon transfection with a functional LT-ß DNA, i.e., clone 12.

Clone 12 lacks an initiating ATG codon, but does possess several CTG initiating codons and hence

15   this expression experiment shows that one or several of the 5' CTG codons can initiate translation. CTG codons are known to serve as initiating sites for translation in several eukaryotic proteins. [M. Kozak, J. Cell. Biol., 115, 4 (1991)]. Similar results were observed

20   using the dual transfection system such that only COS cells receiving both LT-α and LT-ß DNA displayed substantial surface LT-α in a FACS analysis.

Northern analysis of II-23.D7 cells showed hybridization of the LT-ß cDNA to a 0.9-1.0 kb mRNA

25   indicating that the cloned cDNA represents essentially all of the transcribed gene. See Fig. 15. The LT-ß gene was expressed at low levels in untreated II-23.D7 hybridoma cells; however, upon cell activation with phorbol ester mRNA levels increased dramatically. See

30   Fig. 16.

Example 11

Homology Between LT-ß and Other Members of the TNF Family Of Lymphokines

Cloning of the cDNA encoding LT-ß revealed
5 that LT-ß is a type-II membrane protein with
significant homology to TNF, LT-α and the ligand for
the CD40 receptor. These proteins are known to bind to
members of the TNF/NGF receptor family. LT-ß, TNF,
LT-α and the ligand for the CD-40 receptor share four
10 regions of sequence conservation in the extracellular
domain. See Fig. 13. These domains are located on the
face of the TNF and LT-α crystal structures and are
likely to be involved in intersubunit interactions.

Example 12

Determination of the 5' End of LT-ß Reveals Several Possible Start Sites

The 5' mRNA sequence was determined by primer
extension analysis. Primer extension analysis revealed
that the transcriptional start site for the LT-ß
20 protein was approximately 7-9 base pairs upstream of
the methionine ATG. Thus, the mRNA possesses at least
3 possible translation start sites, the Met-1, Leu-4
and Leu-6 codons. Transient experiments with clone 12
showed that one or both of the Leu-4 or Leu-6 start
25 sites is functional. The 5' mRNA sequence was verified
by determining the LT-ß genomic sequence using a cosmid
clone 031A, described above, and dideoxynucleic acid
sequencing.